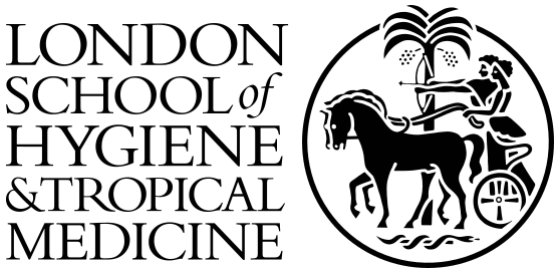

The epidemiology of paediatric diarrhoeal disease and *Shigella* infections in Ho Chi Minh City, Vietnam

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Declaration by candidate

I, Corinne Nicholson Thompson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Shigella is an enteric pathogen that is the most common bacterial cause of dysentery globally, most frequently infecting children in developing countries. Resistance to common antimicrobials is now widespread and is beginning to make the management of this often-severe infection very challenging. Vaccination offers a realistic option for preventing and controlling shigellosis, yet several critical questions need to be answered before a successfully licensed vaccine can be introduced. Ho Chi Minh City (HCMC), in Vietnam, is a rapidly industrialising urban setting with a high burden of paediatric diarrhoeal disease and is representative of many similar regions globally.

Through the structure of a community cohort, the incidence of diarrhoeal disease in children under the age of five in HCMC was found to be 70/100 child years, indicating that diarrhoea remains a significant cause of paediatric morbidity in this location. Furthermore, children living at low elevation in the centre of the city were found to be at increased risk of reported diarrhoeal disease during periods of higher temperature and flooding, highlighting a particular community at risk. This work also documents *Shigella* as a common cause of dysentery in both hospital and the community in HCMC, with community-based incidence estimated to be 1.5/100 child years in 2-5 year olds.

Resistance against a variety of antimicrobials in *Shigella* was detected, and organisms harbouring mutations against fluoroquinolone activity were found to survive for longer periods in the presence of ciprofloxacin *in vitro,* suggesting a potential epidemiological advantage against sensitive strains. Finally, the half-life of maternal immunity against the O-antigen of *S. sonnei* was found to 43 days and by five months of age less than half of children in HCMC have any circulating maternal protection. Work from this thesis will help inform future vaccine rollout efforts and fills important gaps in the current literature surrounding this increasingly challenging infection.

DEDICATION

To Daniel Benjamin Wright (1987-2012) for illuminating the true meaning of stochasticity.

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LIST OF ACRONYMS

ADB	Asian development bank
ALT	Alanine aminotransferase
AMP	Ampicillin
AMR	Antimicrobial resistance
AST	Aspartate aminotransferase
AUG	Amoxicillin-clavulanic acid
AZM	Azithromycin
CAZ	Ceftazidime
CH1	Children's Hospital 1, Ho Chi Minh City
CH2	Children's Hospital 2, Ho Chi Minh City
CHL	Chloramphenicol
CI	Confidence interval/cumulative incidence
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CRF	Case report form
CRO	Ceftriaxone
CYO	Child years of observation
EAEC	Enteroaggregative *E. coli*
EBK	Empirical Bayesian Kriging
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme Linked Immunosorbent Assay
EPEC	Enterpathogenic *E. coli*
EPI	Expanded Programme on Immunization
ERY	Erythromycin
ESBL	Extended-spectrum beta lactamase
ETEC-LT/ST	Enterotoxigenic *E. coli* heat labile/heat stable
EU	ELISA units

LIST OF ACRONYMS cont.

FcRn	Neonatal Fc receptor
FCT	Fever clearance time
GAT/GA	Gatifloxacin
GE	Gastroenteritis
GEMS	Global Enteric Multicenter Study, University of Maryland
GMT	Geometric mean titer
HCMC	Ho Chi Minh City
HCT	Haematocrit
HGT	Horizontal gene transfer
HR	Hazard ratio
HTD	Hospital for Tropical Diseases, Ho Chi Minh City
HUS	Haemolytic uremic syndrome
HVH	Hung Vuong Maternity Hospital, Ho Chi Minh City
ICU	Intensive care unit
IL	Interleukin
Ipa	Invasion plasmid antigen
IQR	Interquartile range
IRB	Institutional Review Board
IRR	Incidence rate ratio
IYO	Infant years of observation
KW	Kruskal-Wallis test
LPS	Lipopolysaccharide
MAL-ED	The Malnutrition and Enteric Disease Study
MDG	Millennium Development Goals
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MSD	Mild to severe diarrhoea
NAL	Nalidixic acid
NoV	Norovirus

LIST OF ACRONYMS cont.

OD	Optical density
OFX	Ofloxacin
OR	Odds ratio
ORF	Open reading frame
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethical Committee
PACF	Partial autocorrelation function
pINV	Invasion plasmid
PMQR	Plasmid mediated quinolone resistance
RBC	Red blood cell
RoV	Rotavirus
RR	Relative risk
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SES	Socioeconomic status
ShET	*Shigella* enterotoxin
STEC	Shiga-toxin producing *E. coli*
Stx	Shiga toxin
SXT	Trimethoprim-sulfamethoxazole
T3SS/TTSS	Type 3 secretion system
WASH	Water sanitation and hygiene
WAZ	Weight for age Z score
WBC	White blood cell count
WHO	World health organization

1 INTRODUCTION

1.1 Diarrhoea

Each year, 10% of all child deaths globally are attributable to diarrhoeal disease [1]. With over 700,000 deaths and1.7 billion episodes annually [1,2], diarrhoea remains a significant public health challenge in young children in industrialising regions [3,4]. The Millennium Development Goals (MDGs) called for a reduction in child mortality by two thirds between 1990 and 2015. Although recent analyses have shown that both mortality and morbidity due to diarrhoeal disease are declining globally [1,5], some regions still report unacceptably high rates in children [5]. Furthermore, antimicrobial resistance (AMR) in some enteric bacterial pathogens is threatening to slow recent progress in tackling this complex and multifaceted syndrome [6,7].

Diarrhoea is defined by the WHO as the passage of unusually loose or watery stools, usually at least three times in a 24 hour period [8]. Severity of a diarrhoeal episode is generally assessed by presence and severity of dehydration, bloody stools, persistent diarrhoea and/or malnutrition [8]. The recent Global Enteric Multicenter Study (GEMS) evaluated the aetiology and population-based burden of moderate to severe diarrhoea (MSD) in >9,000 children in seven developing countries for global policy planning purposes [9]. They define MSD as diarrhoea accompanied by either (1) dehydration to a degree that the child's survival would likely depend on access to life-saving rehydration fluids or (2) evidence of inflammatory destruction of the intestinal mucosa [10].

Diarrhoeal disease in young children can be due infection with a number of pathogens, the aetiology of which is dependent on factors such as age, season and nutrition status [3,4,11,12]. Rotavirus is the most common cause of diarrhoeal disease in the first two years of life globally [3,4]. Prior to introduction of rotavirus vaccines, roughly 450,000 children died each year of rotavirus diarrhoeal disease [13]. Yet after successful endorsement by WHO in the late 2000s, 77 countries now include rotavirus vaccine in their national immunisation programs and consequently, reported rotavirus incidence has fallen dramatically [14,15]. The nature of this success draws attention to the relative lack of progress against other diarrhoeal aetiologies, particularly those causing dysentery [16], a syndrome associated with structural and functional intestinal damage as well as greater endogenous protein loss compared to non-dysenteric diarrhoea [17]. Pathogens known to cause bacillary dysentery in children under five years of age include primarily *Shigella* spp. but also *Campylobacter* spp. and enteroinvasive *Escherichia coli* (EIEC) [3,4].

1.2 *Shigella*

Shigella are a genus of faecal-orally transmitted Gram-negative enteric bacteria of the Enterobacteriaceae family that are responsible for >100,000 deaths and seven million disability adjusted life years (DALYs) annually [18,19]. Of all of the bacterial pathogens that cause diarrhoea, *Shigella* is of particular concern for several reasons. First, its low infectious dose (as few as 10-500 organisms) [20] allows it to spread quickly via person-to-person contact in crowded settings with poor hygiene, highlighted by outbreaks in locations such as daycares and refugee camps [21–24]. Secondly, the clinical severity of *Shigella* can result in life threatening infections and subsequent growth retardation, particularly in children [25,26]. Lastly, *Shigella* have displayed remarkable levels of antimicrobial resistance (AMR) [27–33], complicating treatment options.

Two recent large-scale studies showed that *Shigella* remains one of the most commonly isolated diarrhoeal pathogens in children aged under five years in industrialising regions of Asia, Africa and South America [3,4]. *Shigella* was among the top four pathogens identified in young children in the GEMS study, and was the most frequently isolated pathogen in children aged 24-59 months [3]. Furthermore, from the Malnutrition and Enteric Disease study (MAL-ED), a cohort study evaluating >2,100 children in eight developing countries for the first two years of life, *Shigella* was responsible for a considerable attributable fraction of diarrhoea (4%, 95% confidence interval (CI): 3.6-4.3%) amongst the 12-24 month olds [4]. Within Southeast Asia, though data are more scarce, *Shigella* is ubiquitous with incidence rates per 1,000 per year estimated to be 4.0, 18.6 and 4.9 for Thailand, Indonesia and Vietnam, respectively [34].

1.2.1 Historical Context

Epidemics of dysentery were very common throughout history, particularly in militaries during times of war as the often unhygienic, overcrowded conditions facilitated rapid bacterial spread [16,35]. *Shigella* was first identified by Dr Kyoshi Shiga in Japan in 1898 following an epidemic in the country that infected >91,000 people with a mortality rate of >20% [36]. Dr Shiga isolated specifically *Shigella dysenteriae*, one of the four species of *Shigella* and the species most often identified in large, explosive outbreaks [24]. In the 1940s, Ewing proposed classifying these species into a new genus called *Shigella* (*S. dysenteriae, S. flexneri, S. sonnei* and *S. boydii*) based on the O antigen only, because *Shigella* lack the flagellar H antigen and capsular K antigen used in typing *E. coli* [37,38]. Within each species of *Shigella* there are a number of serotypes based on the diversity in structure of the terminal O polysaccharide of the lipopolysaccharide (LPS), a primary

Shieglla virulence factor [39,40]. Within *S. dysenteriae* there are 15 serotypes, within *S. flexneri* there are 14, within *S. boydii* there are 20 and *S. sonnei* only has 1 serotype [41].

Of the four species of *Shigella*, *S. sonnei* and *S. flexneri* are most prevalent globally [34,42]. *S. sonnei* is traditionally isolated in resource-rich countries, whereas *S. flexneri* is generally associated in industrialising regions [43,44], though this distribution is thought to be changing [45]. While the two species largely share a similar clinical phenotype and epidemiological profile [31], the differences between them are only beginning to be understood. Phylogeographically the two species have distinct histories. The current global population of *S. sonnei* is now considered to have emerged from a single clone after the acquisition of AMR determinants, dispersing globally from Europe and replacing local strains within the last 500 years [45]. *S. flexneri*, however, has been recently shown to persistently colonise regions for long periods of time, ranging from decades to centuries [46]. Local selective pressure due to antimicrobial use is thought to be driving the microevolution of *S. sonnei* [47], whereas this appears not to be the case for *S. flexneri* [46]. Such insights suggest that the epidemiology of *Shigella* is changing in many regions and will continue to do so in the future, warranting a deeper understanding of the epidemiology of the pathogen in regions currently experiencing shifts in *Shigella* species.

AMR amongst *Shigella* is known to be elevated in endemic areas [48,49], including Southeast Asia [27,50–52]. Yet a growing number of recent reports from countries such as the US, South Korea and the Republic of Ireland indicate importation of fluoroquinolone-resistant *Shigella* infections from endemic regions, often with subsequent domestic transmission [53–55]. Furthermore, an epidemic of azithromycin-resistant shigellosis has recently been documented in Europe and North America among men who have sex with men (MSM) [56]. Such trends indicate that shigellosis, particularly AMR strains, is becoming a public health concern not only in industrialising regions but globally.

1.2.2 Clinical Presentation

The symptoms of shigellosis can range from mild watery diarrhoea to severe inflammatory bacillary dysentery; severity is suggested to correspond to bacterial load [57]. After an incubation period of 12 hours to two days, symptoms appear abruptly and include a short period of watery diarrhoea with intestinal cramps and general malaise, followed by fever, tenesmus and eventual emission of bloody, often mucopurulent stools [25]. Damage to the gut mucosal surface results in enterocyte death and the

characteristic mucosal ulcerations of bacillary dysentery [58]. The infection can progress to life-threatening in the immunocompromised, malnourished, very young or if adequate medical treatment is not available [16,59–61]. Death in early stages of the disease is due most often to septicaemia, toxic megacolon or renal failure [62–64]. Bacteremia is rare but has a high mortality rate when reported [25,65,66].

Shigella infections are associated with poor physical development in children, including decreased linear growth [26]. Repeat and persistent diarrhoeal infections are known to have dramatic effects on intestinal absorption, resulting in intestinal enteropathy and malnutrition [67]. This, in turn, renders the child more susceptible to future diarrhoeal infections and can lead to impaired physical and cognitive growth and increased risk for long-term chronic morbidities later in life, particularly in impoverished areas [68,69].

1.2.3 Pathogenesis

The *Shigella* are recently emerged clones of *Escherichia coli*, which have adopted an intracellular, pathogenic lifestyle [70]. This shift to intracellularity was due to the recent and independent acquisition of an invasion plasmid (pINV), the first of which were acquired ≤300,000 years ago [70,71]. *Shigella* has undergone purifying selection as a function of niche specialisation as it has lost traits such as catabolic pathways and flagellar proteins that are no longer required once surviving intracellularly [70–72]. *Shigella* are also able to acquire and transfer plasmids that are critical for pathogenesis, the accumulation of which were important steps in the diversification from their non-pathogenic *E. coli* ancestors [59].

The virulence plasmid and associated virulence genes are an essential determinant of all *Shigella* as they provide for tissue invasion as well as an intracellular lifestyle [73]. These virulence genes are under tight control of a regulator network that responds to environmental changes. The major trigger inducing the expression of the virulence plasmid is a temperature shift to 37°C [74]. Once ingested, *Shigella* invades the gut epithelial cells through the antigen-sampling microfold cells (M cells) in the colon [75,76], as shown in Figure 1. The bacteria are then phagocytosed by resident macrophages and dendritic cells in the lymphoid follicle. *Shigella* evades degradation by rapidly escaping from the phagocytic vacuole in the macrophage by destroying the phagosomal membrane via the invasion plasmid antigen (Ipa) IpaB [77]. Following escape, *Shigella* induces apoptotic cell death of the macrophage [78]. This leads to the release of proinflammatory cytokines (interleukin (IL)-1β and IL-18) which stimulate a strong intestinal inflammatory response [59].

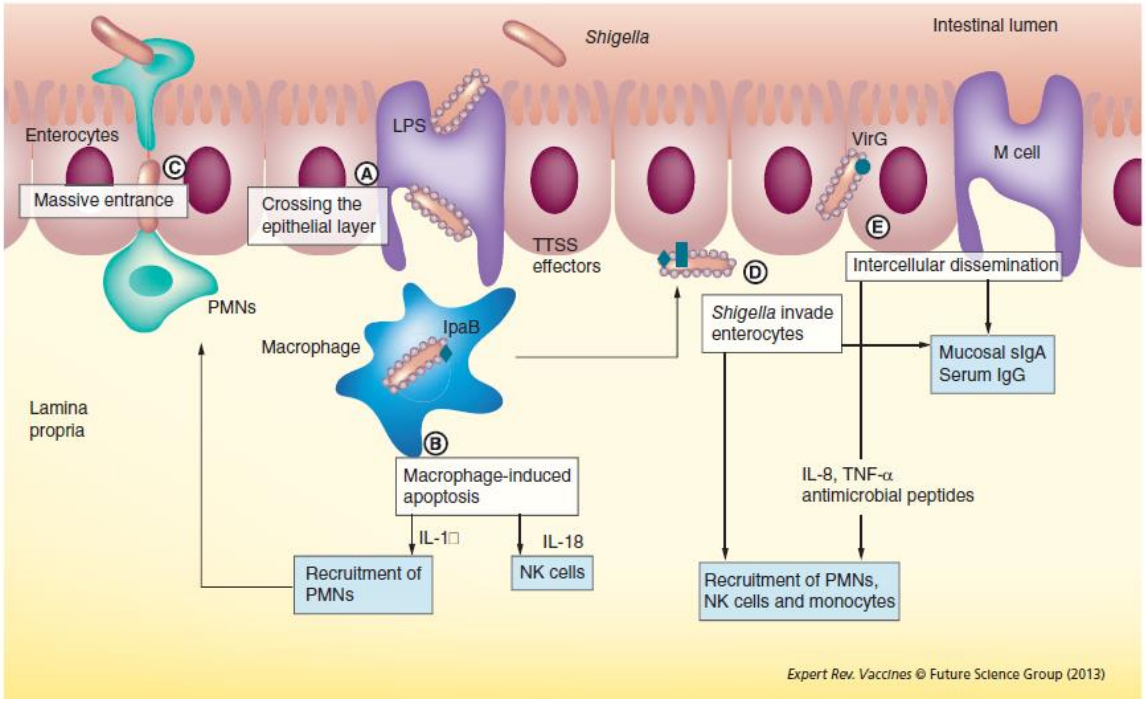

Figure 1: *Shigella* invasion of gastrointestinal epithelium. Ipa: Invasion plasmid antigen; LPS: lipopolysaccharide; NK: Natural Killer; PMN: Polymorphonuclear cell; sIgA: Secretory IgA; TTSS: Type 3 secretion system. Take from: Camacho AI, Irache JM, Gamazo C. Recent progress towards development of a Shigella vaccine. Expert Rev. Vaccines 2013; 12:43–55.

Once the organism has escaped the macrophage, the bacteria then gain access to the basal surface of the enterocytes, where it uses a type 3 secretion system (T3SS) to invade the host cell and replicate in the cytoplasm [73]. To invade the enterocyte, the needle of the T3SS (formed of proteins IpaB, IpaC and IpaD) is inserted into the host cell and forms a pore through which other proteins are transported [79]. T3SS enables the translocation of ~25 effector proteins from the bacterial cytoplasm directly to the eukaryotic host cell where they interfere with a variety of host cell processes [80,81]. After T3SS insertion, the membrane of the enterocyte starts to ruffle due to the effector-mediated induction of actin polymerisation and forms a macropinocytic pocket that encloses the bacteria [59]. The bacteria is then trapped in a phagosome after uptake, which it quickly lyses and escapes into the cytosol via effector proteins IpaB, IpaC and IpaD [82,83].The cytoplasm of the enterocyte is the main replicative niche for *Shigella*, which is relatively unique among enteric bacteria [70].

Shigella do not have flagella and are therefore non-motile. They instead rely on an elaborate mechanism to hijack the host cytoskeleton via the plasmid encoded protein IcsA which localises to one pole of the bacterium [84]. IcsA (also known as VirG) initiates localised actin polymerisation, which results in actin cross-linking and contraction and provides a propulsive force which is energised by ATP [85]. Intracellular motility is also

dependent on the T3SS substrate IcsB, which protects the bacteria from being recognised and entrapped by the host cell autophagy machinery [86]. IcsA also allows for the formation of extracellular protrusions that permit cell to cell spread as well as intracellular microfilament movement [84]. *Shigella* is therefore capable of dissemination to neighbouring host cells while avoiding exposure to the extracellular host immune defences [59,84].

The immune response to *Shigella* during the infection process plays a critical role in the pathogenesis of the bacteria. A large part of the mucosal inflammatory response in *Shigella* infections is mediated by sensing of bacterial peptidoglycan in the epithelial cells [87]. IL-8 is the major chemokine mediating the inflammatory burst and leads recruitment of polymorphonuclear cells that cross the epithelial layer by impairing tight junctions between the enterocytes, which in turn allows a massive entry of bacteria [88,89]. This translocation of the bacteria from the intestinal lumen to the subepithelial lamina propria of the colon is considered to be a primary *Shigella* virulence mechanism as it provides for direct access for *Shigella* to breach the enterocyte [59]. Yet *Shigella* must carefully control the immune response for successful invasion.

Shigella can modulate the immune response in a number of ways [87]. For example, *Shigella* are able to downregulate the expression of antimicrobial peptides, which are important antibacterial effectors constantly released from the mucosal surfaces of the gastrointestinal tract [90]. Additionally, an effector called OspG can prevent activation of the NF-κB pathway, the heart of the signalling cascade leading to the mucosal immune response, to negatively control the host innate immune response upon invasion of the epithelium [91]. This initial down-regulation allows *Shigella* to interact with the intestinal epithelium to facilitate colonisation and invasion by an initially low number of luminal bacteria [91]. However, once successfully inside of an enterocyte, upregulating inflammation is then advantageous for *Shigella* as it allows for a large number of bacteria to enter the lamina propria via broken tight junctions between enterocytes [87].

1.2.3.1 Toxins

Shigella can produce several enterotoxins, known as *Shigella* enterotoxin 1 (ShET1) and ShET2 that alter electrolyte and water transport in the small intestine during the initial watery phase of the disease [41]. ShET1, encoded by the *set* genes located on the chromosome of many clinical isolates of *S. flexneri* 2a, induces intestinal fluid accumulation and cause net fluid secretion [92–94]. ShET2 is found in many *Shigella* serotypes [95] and is encoded by the *sen* gene located on the large invasion plasmid

[96]. Both of these toxins continue to serve as targets for attenuating mutations in vaccine candidates [97–99].

Shiga toxins (Stx) are potent protein-synthesis inhibitors that target the vascular endothelium, including the colon, kidneys and central nervous and can lead to hemolytic uremic syndrome (HUS) [100] as well as other life threatening complications [101,102]. Furthermore, antimicrobial therapy for infections involving Stx have been associated with an increased risk of HUS [103]. Stx1 is a cytotoxic protein made by primarily *S. dysenteriae* 1 and can also be found Shiga-toxin producing *E. coli* (STEC) [101]. In *S. dysenteriae* 1 the genes encoding the toxin (*stxA* and *stxB*) are found on the chromosome [104]. Stx1 is traditionally not found in other serotypes or species of *Shigella* [59] and is not required for virulence [16]. However, alarmingly, there are recent reports of phage-mediated Stx-producing *S. flexneri* and *S. sonnei* infections from a disparate number of locations, including Haiti and the USA [105,106].

1.2.3.2 Shigella and Enteroinvasive E. coli (EIEC)

EIEC and *Shigella* are taxonomically indistinguishable at a species level [107] and share similar pathogenic mechanisms and biochemical characteristics [59,108]. The two bacteria are traditionally distinguished by minor biochemical properties [107]. Though EIEC normally elicits watery diarrhea [109], it can also cause dysentery [110]; the infective dose of EIEC is far larger (~10^8 organisms) [111,112] compared to *Shigella* (10-500 organisms) [20]. Like *Shigella*, EIEC strains have a virulence plasmid that allows for invasion of epithelial cells and cell-to-cell dissemination [113]. While EIEC and *Shigella* share a large number of virulence genes [107,113], phylogenetic analyses suggest that *Shigella* and EIEC evolved independently and EIEC as a group cannot be considered as the ancestor to *Shigella* [108]. However, recent whole genome sequencing analyses have provided evidence to suggest that *Shigella* belong in the *E. coli* genus and share the same pool of genes. Therefore, many argue that the *Shigella* genus should be moved back within the species of *E. coli*, classified as EIEC and renamed using the common O antigen naming system of *E. coli* [108,114,115]. Yet due to its clinical severity, others have favoured the traditional nomenclature separating the *Shigella* genus from *E. coli* [107].

1.2.4 Antimicrobial Resistance

AMR among *Shigella* and other Gram-negative pathogens represents one of the challenges currently facing paediatricians in developing regions [6]. Though the WHO explicitly recommends that antimicrobials not be used routinely for the treatment of

paediatric diarrhoea [8], the exception is in the case of shigellosis, with current guidelines recommending the fluoroquinolone ciprofloxacin, followed by second line therapies pivmecillinam, ceftriaxone and azithromycin [116]. Reduced susceptibility and often full resistance to these antimicrobials is now reported amongst *Shigella* isolates in many regions globally [27–33,48,49,117,118].

The mechanisms of antimicrobial resistance in *Shigella* are diverse. Several mechanisms of quinolone resistance, for example, have been identified amongst *Shigella* and the Enterobacteriaceae family. The quinolones and fluoroquinolones target the bacterial enzymes DNA gyrase (*gyrA* and *gyrB* genes) and topoisomerase IV (*parC* and *parE* genes) that are essential for bacterial replication. The bacteria may acquire chromosomal mutations in the quinolone resistance determining region (QRDR) that includes *gyrA*, *gyrB*, *parC* and *parE*, altering the target of the drug [30,119,120]. *Shigella* can also acquire plasmid-mediated quinolone resistance (PMQR) such as *qnr* genes, which encode pentapeptide repeat proteins that bind to and protect DNA gyrase and topoisomerase from the action of fluoroquinolones. Plasmids containing genes for an aminoglycoside acetyltransferase that is capable of acetylating and reducing the activity of fluoroquinolones (*aac(6')-Ib-cr)* as well as those containing genes upregulating efflux pump activity (*tolC)* have also been documented in *Shigella* [121–125]. The MIC against fluoroquinolones generally rises with an increasing number of resistance determinants [126].

1.2.5 Immune Response & Vaccines

During infection with *Shigella*, the host adaptive immune response targets the LPS O-side chain, the major bacterial surface antigen, which is serotype specific [127–133]. Infection with wild-type or experimental *Shigella* confers protective immunity and prevents disease during subsequent exposures of the same serotype [130,134–136] with one cohort study in Chile estimating 72% protection against homologous serotype reinfection [127]. Although not considered a definitive correlate of immunity [137], it has been shown that serum anti-LPS is a strong marker of acquired immunity and that lack of serotype specific antibody is associated with an increased risk for symptomatic disease [133,136,138–140]. Both serum IgG and IgA antibodies directed against the O-antigen are particularly important and appear 1-2 weeks after primary exposure [132,133,136]. [141]. In terms of a cell-mediated response, evidence suggests that $CD8^+$ T cells are not required for protective immunity because they fail to be primed as *Shigella* can impair the migration pattern of $CD4^+$ T cells [142,143].

The debate as to whether protection is mediated predominantly via secretory IgA (sIgA) or serum IgG or both is contentious [41]. LPS-specific sIgA is the major mucosal antibody induced upon natural infection and can survive, anchored by mucus to enterocytes, for extended periods in the harsh gastric environment and likely prevents colonisation and *Shigella*-associated inflammation responsible for tissue destruction [137,144,145]. Gut-derived O-specific IgA antigen secreting cells are detected in peripheral blood 7-10 days after exposure, and are believed to represent a pool of transiently migrating antigen-specific B cells with the capacity to home to the gut [141]. Measurements of sIgA in mucosal secretions can be variable, however [146]. *Shigella* is also likely inactivated by systemic IgG leaked into the intestinal lumen, possibly through complement-mediated lysis [141,147]. Finally, there is known to be a significant rise in anti-Ipa antibodies after natural exposure and experimental infections and in endemic areas anti-Ipa titers have been shown to increase with age [132,138,139]. It is thought that in addition to anti-LPS antibodies, anti-Ipa antibodies also play a significant role in protection [141].

Vaccination still offers the greatest hope of an effective and sustainable strategy to control shigellosis and significantly reduce the burden of disease [148,149]. The WHO has listed *Shigella*, along with enterotoxigenic *E. coli* (ETEC), among the top candidates on its vaccine development priority list [67,149]. Although a considerable number of trials have been conducted on a variety of *Shigella* vaccine candidates since the1960s [150], none have yet proved both safe and effective [41].

1.2.5.1 Conjugate and subunit vaccines

As protection against *Shigella* is serotype specific, one major route of vaccine development has focused on the immunogenicity of the serotype specific LPS [149]. Parenteral conjugate vaccines pursuing this serotype-specific protection strategy linking *Shigella* LPS to a carrier protein have received much attention [147], and have been trialled with some success [151,152]. Although LPS alone is poorly immunogenic [153], when covalently coupled to protein carriers it can induce a stronger and longer-lasting T cell-dependent immune response [154]. A multivalent candidate including *S. sonnei* and *S. flexneri* serotypes 2a, 3a and 6 and possibly *S. dysenteriae* 1 would provide protection against the bulk of infections worldwide [141,155], with cross-protection against an additional 11 *S. flexneri* serotypes [42].

One candidate vaccine, *S. sonnei* LPS conjugated to the *Pseudomonas* exoprotein A (rEPA) was shown to be efficacious in Israeli soldiers [156,157] but did not show efficacy

in Israeli children under two years of age in further trials [152]. The Walter Reed Army Institute of Research has developed a subunit candidate called Invaplex using Ipa proteins linked to serotype-specific LPS which has proven safe and immunogenic after intranasal delivery in healthy volunteers [158,159]. Invaplex is currently in a phase 1 trial in healthy adults [160]. Additionally, candidate conjugate vaccines using synthetic LPS conjugated to tetanus toxoid have also been developed in recent years as well, although are yet to be experimentally tested in humans [161,162]. It has been suggested that conjugate vaccines would be maximally protective when administered to individuals previously mucosally primed to elicit mucosal as well as systemic responses [149].

1.2.5.2 Live attenuated vaccines

Molecular engineering of *Shigella* genomes led to the generation of live, attenuated, oral *Shigella* vaccine candidates with defined deletions and mutations in specific virulence or metabolic genes. This strategy delivers a high level of antigen exposure, typically induces a large immune response but can prove to be reactogenic in addition to the risk of reversion [137]. The Center for Vaccine Development at the University of Maryland has developed several iterations of an attenuated *S. flexneri* 2a strain, first generating a Δ*virG* (alternate name for IcsA) Δ*aroA* candidate that inhibited intracellular movement and disrupted a metabolic process which proved too reactogenic [163], followed by a candidate with similar motility and metabolic mutations (Δ*virG*, Δ*guaBA*) in addition to mutated ShET toxin genes (Δ*sen*, Δ*set*) which proved to be over-attenuated [164]. They then produced a Δ*guaBA* (metabolism), Δ*sen*, Δ*set* mutant with an encouraging safety and efficacy profile [99].

Meanwhile, the Walter Reed Army Institute of Research with Johns Hopkins University have developed an attenuated *S. flexneri* 2a candidate with impaired intracellular movement (Δ*virG*) which was safe and effective in North American volunteers, but failed to induce an immune response in Bangladeshi children [165,166], potentially due to low availability of intestinal iron in the Bangladeshi population [149]. However, 'second generation' Δ*virG* mutants with additional deletions in ShET genes are under development and early clinical evaluation [167–170]. Finally, a *S. sonnei* candidate with Δ*virG*, Δ*sen*, Δ*set* has proved to be safe and immunogenic in primates [170].

1.2.5.3 Inactivated whole cell vaccines

Although historically difficult to balance immunogenicity and safety in killed whole cell vaccines [167], formalin-inactivated *S. sonnei* and *S. flexneri* candidates have been

shown to be safe and immunogenic in North American volunteers [171,172]. Efficacy studies are now required [141].

1.2.5.4 Vaccine challenges

Several barriers continue to slow the pace of *Shigella* vaccine development including undefined correlates of immunity, a lack of a good small animal model, difficulty in providing broad coverage and continuing economic and political challenges [141,173]. Enteric vaccines as a whole are hindered due to limited knowledge of gut immune function in children, lack of mucosal adjuvants and a limited understanding of why mucosal vaccines fail in industrialising regions [145]. Efficacy of enteric vaccines in children in developing countries, particularly mucosal vaccines, is notoriously low compared to many developed countries [174–176]. Environmental enteropathy is thought to contribute substantially to reduced immunogenicity in children from poor areas, with explanations including blunted villi, heightened inflammation due to concurrent parasite infection, small bowel overgrowth, malabsorption, caloric and micronutrient deficiencies (including vitamin A and zinc) and increased gut permeability and bacterial translocation [12,145,177,178]. Furthermore, there is likely to be interference from maternal antibodies, acquired both transplacentally and via breastmilk [179–181].

Parenteral vaccines are generally poor inducers of mucosal immunity and less effective against enteric pathogens [137]. However, mucosal vaccines face great challenges in addition to environmental enteropathy of a child host in a developing country. Mucosal immune regulation is designed to prevent inadvertent immune responses to dietary or environmental antigens [173,182], and therefore stimulating it sufficiently is often challenging. Additionally, mucosal vaccines can be diluted in mucosal secretions, captured in mucus gels or attacked by proteases so relatively large doses of vaccine are required, though it is not possible to determine how much vaccine crosses the mucosa after administration [183]. A mucosal adjuvant and delivery system would be advantageous [145], particularly as alum cannot be used orally [137]. Furthermore, use of antimicrobials, anti-inflammatory agents, probiotics, deworming pills, vitamin A or drugs modulating gut permeability may improve mucosal vaccine performance in industrialising countries [145,178].

Optimal protection against *Shigella* will likely require both systemic and mucosal responses and the multifactorial nature of virulence indicates the vaccine will likely need to have a number of antigens [137]. An ideal *Shigella* vaccine would be temperature-resistant and administered as a single dose infant vaccine integrated into the Expanded

Programme on Immunization (EPI) [177], although oral vaccination is known to be most effective when given in multiple doses [184]. Oral vaccines induce stronger mucosal immune responses and tend to be more accepted by parents than parenterally delivered vaccines. Mucosal vaccines also reduce issues surrounding needles and highly trained healthcare professionals [178,183,185], are generally easier to manufacture [145], and make mass campaigns easier [178]. However, live attenuated *Shigella* vaccines have suffered from problems with reactogenicity [163,164]; it has been suggested that the ability of a vaccine to invade may be a prerequisite for induction of effective immunity, which requires a careful balance between immunogenicity and reactogenicity [182].

1.3 Vietnam: background and diarrhoeal disease burden

As recently as the 1980s**,** Vietnam was considered one of the poorest countries on earth [186]. Yet it has emerged from war and famine to make remarkable progress in economic development since it reopened for international investment in the early 1990s [187,188]. It has a GDP per capita on par with the Philippines and Indonesia [189], and was recently upgraded from a low income country to a lower-middle income country by the World Bank in 2012 [190]. Vietnam is now a rapidly industrialising country with an urbanising population of 90 million people and a Gross National Income (GNI) per capita of US $1,740 [191]. Ho Chi Minh City (HCMC) is the largest city in Vietnam, with an official population of approximately eight million people [192]. HCMC is located in the tropical south of the country, with an average annual temperature of 28^{o}C and total yearly rainfall of almost two meters [193], which falls during the rainy season of May-November.

Vietnam on the whole has made excellent progress in healthcare in recent decades, with life expectancy in men and women rising from 66 and 72 years, respectively, in 1990 to 72 and 80 years, respectively, in 2013 [5]. Additionally, under-five mortality has fallen from 51/1000 live births in 1990 to 24/1000 live births in 2012 [194]. Behind this rapid advancement lies a growing wealth disparity, however, particularly among rural and urban poor populations [188,195]. Under-five mortality and prevalence of children who are underweight, for example, are highest in the poorest quintile of the population [188,196]. Vietnam is also currently undergoing an epidemiological transition whereby deaths due to non-communicable disease are growing yet those due to infectious causes are still significant in number [5,197]. Such a complex transition has dramatic implications for public health policy planning and resource allocation in the country.

Healthcare expenditure makes up roughly 6% of the gross domestic product of Vietnam, with national public health insurance accounting for 30% and private expenditure making up 70% in 2014 [198]. Though the government are aiming to provide free nationalised health insurance for all citizens [199], as was done prior to 1989 [198], only 60% of the population was covered in 2011 [199]. In June 2014 the national assembly passed an insurance law designed to make participation compulsory [200], though Vietnam still relies heavily on out-of-pocket payments to finance health care with a high fraction of Vietnamese households experiencing catastrophic and/or impoverishing out-of-pocket payments [201]. Public hospitals in Vietnam are often overcrowded, particularly in urban areas, and struggle to cope with high patient demand. The government legalised private hospitals in 1999 in an effort to reduce pressure on state-funded hospitals, though they contribute less than 5% of all hospital beds in the country [202].

EPI vaccinations have made a substantial impact on childhood mortality and have been very cost effective in Vietnam [203], with an estimated 95% of children covered by 3 doses of diphtheria-tetanus-pertussis vaccine and 94% covered by two doses of measles vaccine in 2014 [204]. The prevalence of childhood malnutrition in Vietnam was estimated to be 15% in 2013, ranking high among Asian countries [205]. From a large, recent study evaluating nutrition across several sites Vietnam, approximately 24% of children aged 5 are stunted (height-for-age Z score <-2) [206]. The HIV prevalence in the country is low, with 0.5% of the adult population aged 15-49 positive [207]. A total of 67% of the Vietnamese population are live in rural areas [191], though the country is rapidly urbanising [208].

Diarrhoea is a leading cause of paediatric morbidity and mortality in Vietnam, which has an estimated diarrhoeal incidence of 11.5/100/year in children under five years [34,194]. A nationwide survey published in 2008 indicated that the burden of shigellosis in Vietnam remains unacceptably high [209]. *Shigella* patients have been shown to present frequently in hospital in HCMC [31] and a population-based study in central Vietnam estimated an incidence of 490/100,000/year in children under five years [34]. Previous work has shown that, as in other developing nations, *Shigella* tends to peak in the summer months during the rainy season [31,209] and is most frequently identified in children aged two to three years in Vietnam [31,34,210]. Spatial risk analysis has identified potentially different ecological niches between *S. flexneri* and *S. sonnei* in central Vietnam [211]. One study exploring a collection of *Shigella* isolates from hospitalised children found that shigellosis patients were likely to live in areas of HCMC associated with high population density and poor sanitation [31].

The species shift from *S. flexneri* to *S. sonnei* has been dramatic in Vietnam. In 1995-96, for example, only 30% of *Shigella* isolates from children admitted to hospital in HCMC were *S. sonnei* compared with 70% by 2007-2008 [31]. It is thought that a multidrug resistant (MDR) *S. sonnei* clone entered Vietnam in the early 1980s and then spread geographically around the country, originating in HCMC and evolving locally due to selection for MDR through gene transfer within the locally available accessory gene pool [47]. Previous work indicates that it is the expansion of a single successful MDR clone of *S. sonnei*, rather than a generalised increase in various strains, which is replacing *S. flexneri* as the predominant species in Vietnam [47].

1.4 Gaps in knowledge

Shigella is a consistent problem in areas of poor sanitation infrastructure and may emerge as a global public health problem due to international travel and AMR. Vaccination offers a realistic option for combating this challenge [148]. However, in order to effectively roll out a *Shigella* vaccine in the future there are still several critical questions that need to be answered. Firstly, reliable community estimates of diarrhoea and *Shigella* incidence in young children in endemic regions, particularly where *S. sonnei* is thought to be emerging, are lacking. Accurately quantifying the burden of disease through active surveillance is necessary to evaluate the future demand for such a vaccine. Secondly, maternal immunity against *Shigella* in infancy is extremely ill-defined. If a future vaccine can be administered at some point in the first year of life in the EPI schedule, it is important to understand the dynamics of maternal immunity to ensure that the timing of a *Shigella* vaccine is appropriate.

In the absence of a licensed vaccine, optimising treatment regimens would provide physicians with a more appropriate way of managing infections. Quantifying the relationship between fluoroquinolone resistance and clinical outcome, for example, may help inform treatment policy. Furthermore, investigating risk factors and potential transmission routes within an urban, industrialising area may shed light on primary routes of transmission in this setting and may illuminate non-vaccine based prevention strategies for controlling this increasingly resistant infection.

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2 AIM, OBJECTIVES, STRUCTURE & CONTRIBUTIONS

2.1 Aim

The principal aim of the thesis is to quantify the burden of both diarrhoea generally and paediatric *Shigella* diarrhoeal infections specifically in HCMC, Vietnam to determine whether a *Shigella* vaccine would be necessary in this setting.

2.2 Objectives

In order to define the burden of diarrhoeal disease and determine the need of a *Shigella* vaccine in HCMC, several major avenues of investigation will be pursued. The specific objectives of the thesis include:

1) Describe the aetiology of diarrhoea in HCMC
2) Define the spatiotemporal trends of diarrhoea in HCMC
3) Quantify the age-specific burden of both diarrhoea and *Shigella* infections in the community in children under five years of age
4) Define the duration of maternal antibody against *Shigella* in infancy
5) Evaluate the impact of fluoroquinolone resistance on clinical outcome of *Shigella* patients

By performing the listed objectives, a greater understanding of the burden of diarrhoea in HCMC will be generated. An accurate, up to date description of the aetiology and burden of diarrhoea, evaluated through hospital-based and communities studies, will provide an evaluation as to the relative importance and frequency of *Shigella* infections in young children in this setting. Such burden estimates will be important for evaluating the necessity of a vaccine. Next, examining maternal antibody dynamics will provide critical information on transfer of antibody from mother to foetus as well as duration of circulation of antibody in the infant. Such data are important to inform any vaccination schedule should a licensed vaccine become available. Finally, in the event that a vaccine is not deemed necessary, developing a more thorough understanding of the risk factors for diarrhoeal disease in the community will allow for generation of non-vaccine based prevention and control measures. Understanding the impact of antimicrobial resistance on therapeutic outcome, for example, will help tailor more appropriate treatment regimens in this setting.

Diarrhoea throughout this thesis refers to the passage of at least three unusually loose stools in a 24 hours period or one bloody/mucoid stool [1]. While MSD is a concern

globally, the majority of diarrhoeal disease seen in hospitals in HCMC does not meet the criteria for MSD (dehydration to a degree that the child's survival would likely depend on access to life-saving rehydration fluids or evidence of inflammatory destruction of the intestinal mucosa [2]) [3].

2.3 Structure and contribution of research papers

This PhD is split into two sections. The first examines the aetiology and epidemiology of diarrhoea generally, both in hospital and in the community in HCMC. This work was conducted to put the burden of *Shigella* infections (described in part two) into context. There are three research papers included in part one. The second section of the thesis investigates the burden and characteristics of *Shigella* infections in Ho Chi Minh City in closer detail. Age specific incidence in the community, duration of maternal immunity and clinical response to fluoroquinolone therapy are discussed to highlight important characteristics of the infection. There are five papers included in part two.

2.3.1 Part 1: General diarrhoea

2.3.1.1 Paper 1

Research paper 1, entitled "*A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam*", was published in the American Journal of Tropical Medicine & Hygiene in 2015. This work is the first detailed description of the aetiology, clinical manifestations, antimicrobial resistance and prescribing habits amongst doctors for both bacterial and viral hospitalised diarrhoea in children under 5 years of age in Ho Chi Minh City, Vietnam.

2.3.1.2 Paper 2

Research paper 2 was published as "*The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam*" in Health & Place in 2015. This paper examines spatiotemporal variation in hospitalised diarrhoeal disease in Ho Chi Minh City and highlights central and southern regions of the city to be at increased localised risk of reported diarrhoeal disease due to temperature and humidity fluctuations.

2.3.1.3 Paper 3

Research paper 3 was published as "*The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study*" in the International Journal

of Infectious Diseases in 2015. This work quantifies the burden of diarrhoeal disease in the first year of life from a birth cohort in Ho Chi Minh City as well as a rural area of the Mekong Delta. This cohort, though it employed passive surveillance for diarrhoeal disease, offers the first community-based incidence estimates of diarrhoeal disease for these locations.

2.3.2 Part 2: *Shigella*

2.3.2.1 Paper 4

Research paper 4 was published as "*The rising dominance of Shigella sonnei: An intercontinental shift in the etiology of bacillary dysentery"* in PLoS Neglected Tropical Diseases in 2015. This review examines reasons for the relative increase in *Shigella sonnei* against *S. flexneri* in areas traditionally dominated by the latter.

2.3.2.2 Paper 5

Research paper 5 was published as "*A cohort study to define the age-specific incidence and risk factors of Shigella diarrhoeal infections in Vietnamese children: a study protocol"* in BMC Public Health in 2014. This paper is a protocol describing a longitudinal cohort study designed to estimate the age specific incidence of *Shigella* infections in the community in Ho Chi Minh City. Methodology behind the active surveillance of diarrhoeal disease is presented along with study rationale, aims and objectives.

2.3.2.3 Paper 6

Research paper 6 has been written in preparation for publication and is titled "*Active surveillance for diarrhoeal disease in the community: incidence of Shigella and other enteric infections in Ho Chi Minh City*". This work details results from the first year of the longitudinal community cohort study and provides age-specific incidence estimates for *Shigella* as well as a variety of additional aetiologies including norovirus, *Campylobacter* and rotavirus infections. Clinical manifestations, basic risk factors and coinfections from the first year of the cohort are discussed in the context of future planned work for data and samples from the study.

2.3.2.4 Paper 7

Research paper 7 is in press as "*The transfer and decay of maternal antibody against Shigella sonnei in a longitudinal cohort of Vietnamese infants*" in the journal Vaccine in 2015. In this paper, the duration of maternal immunity against *S. sonnei* is quantified for

the first time and longitudinal measurements of anti-*S. sonnei* IgG allow estimation of when an *S. sonnei* vaccine should be administered if a successful candidate is licensed in the future.

2.3.2.5 Paper 8

Research paper 8 is in press as "*The clinical implications of reduced susceptibility to fluoroquinolones in paediatric Shigella sonnei and Shigella flexneri infections*" in the Journal of Antimicrobial Chemotherapy. Here, we evaluate differences in clinical response between *S. sonnei* and *S. flexneri* in treatment with fluoroquinolones for the first time. This paper also fills an important gap in the literature by examining *in vitro* growth of *Shigella* in various concentrations of fluoroquinolones.

2.4 Contributions by candidate and others

Unless otherwise noted, I, Corinne Thompson, have performed all analyses and written all manuscripts included in this thesis. I have not performed any work in the laboratory and have shared responsibilities in setting up some of the studies that are included herein. I have independently performed all literature reviews and have written the introduction and discussion sections of the thesis. Specific contributions to each chapter are listed below.

Paper 1: A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam

> I am co-first author, along with Dr My VT Phan. Stephen Baker designed the study. Dr Phan set up the study at the sites and oversaw sample and data collection at the hospitals. Dr Phan performed all laboratory work. I was responsible for all data cleaning, management and analysis. I corrected errors in the data from source documentation and generated the finalised dataset for analyses. Furthermore, I developed all of the STATA code for analysis and R code for figures. Dr Phan and I both generated the first draft of the manuscript. I created all final tables and figures and was the primary author involved in manuscript preparation and submission.

Paper 2: The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam

I am co-first author, along with Dr Jon Zelner of Columbia University. Stephen Baker, Dr Zelner and I designed the study collaboratively. I collated and cleaned all of the hospital data (>500,000 records) including overseeing the extensive cleaning of the geographic data. I also was responsible for securing the climate data from the Vietnamese government. I performed all geostatistical mapping analyses. Dr Zelner developed the R code for the mixed effects model and goodness of fit evaluations. I wrote the manuscript, prepared all figures and tables and was responsible for the submission procedures.

Paper 3: The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study

I am co-first author, along with Dr Katherine Anders. Dr Anders set up the original birth cohort study in 2009 and oversaw the management, data and sample collection and data management of the entire cohort, including field work and laboratory analyses. A number of individuals were responsible for the laboratory data (Ms Le Thi Phuong Tu, Ms Tran Thi Ngoc Dung and Ms Nguyen Thi Van Thuy). Dr Anders and myself collaborated to design the study together. I cleaned and collated all demographic and descriptive data relevant to the diarrhoea study from the large database. We both were responsible for cleaning and linking diarrhoeal episode data to illness visit records. I developed the STATA code and performed the regression analyses. I also performed the geospatial cluster analysis. I made all tables in the manuscript and Dr Anders generated Figures 1 and 2 for the manuscript. I drafted the manuscript and was responsible for the submission process.

Paper 4: The rising dominance of *Shigella sonnei*: An intercontinental shift in the etiology of bacillary dysentery

I am first author, performed all literature review, generated all figures and wrote the manuscript. Pham Thanh Duy performed the antimicrobial susceptibility testing and gene content analyses.

Paper 5: A cohort study to define the age-specific incidence and risk factors of *Shigella* diarrhoeal infections in Vietnamese children: a study protocol

I am first author. I worked with Stephen Baker on conceiving the design of the study, wrote the protocol and patient information materials, and oversaw the ethical approval process. I was heavily involved in study set up at the two

participating hospitals, including data and sample collection and management. Ms Le Thi Quynh Nhi was responsible directly for field work. I wrote the manuscript, generated all figures and was responsible for the submission process.

Paper 6: Active surveillance for diarrhoeal disease in the community: incidence of *Shigella* and other enteric infections in Ho Chi Minh City

I am first author. Stephen Baker and I worked together to design the study. I wrote the first draft of the protocol, was heavily involved in ethical approval processes, led the daily management of the cohort study and oversaw data and sample management. I was responsible for database cleaning and development of analysis plans. I wrote the STATA code for all analyses and the R code for all figures. Ms Ha Thanh Tuyen and Mr Voong Vinh Phat performed all laboratory work. Ms Le Thi Quynh Nhi was directly responsible for field work. I wrote the manuscript.

Paper 7: The transfer and decay of maternal antibody against *Shigella sonnei* in a longitudinal cohort of Vietnamese infants

I am first author. I led the management of the cohort study, oversaw data collection and management and cleaned all data for this manuscript. I developed the analysis and code in STATA and R, with assistance from Dr Phung Khanh Lam in the OUCRU Biostatistics Department. Ms Le Thi Phuong Tu performed all ELISAs. I made all tables and figures, wrote the manuscript and was responsible for submission of the work.

Paper 8: The clinical implications of reduced susceptibility to fluoroquinolones in paediatric *Shigella sonnei* and *Shigella flexneri* infections

I am first author. Stephen Baker designed the study. I cleaned and analysed the primary data. I wrote the code for analysis in STATA and R with assistance from Dr Nguyen Duc Anh in the Biostatistics Department at OUCRU. Ms Ha Thanh Tuyen was responsible for all laboratory work. Dr Ha Vinh was responsible for the randomised controlled trial and field work. I made all tables and figures for the work. I wrote the manuscript and was responsible for the submission.

2.5 References

1. World Health Organization. Treatment of Diarrhoea: A manual for physicians and

other senior health workers. Geneva: 2005. Available at: whqlibdoc.who.int/publications/2005/9241593180.pdf.

2. Kotloff KL, Blackwelder WC, Nasrin D, et al. The Global Enteric Multicenter Study (GEMS) of diarrheal disease in infants and young children in developing countries: epidemiologic and clinical methods of the case/control study. Clin. Infect. Dis. **2012**; 55 Suppl 4:S232–45.

3. Thompson CN, Phan Vu Tra M, Nguyen Van Minh H, et al. A Prospective Multi-Center Observational Study of Children Hospitalized with Diarrhea in Ho Chi Minh City, Vietnam. Am. J. Trop. Med. Hyg. **2015**; 95:1045–1052.

3 RESEARCH PAPER 1: A prospective multi-center observational study of children hospitalized with diarrhea in Ho Chi Minh City, Vietnam

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RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	American Journal of Tropical Medicine and Hygiene		
When was the work published?	2015		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	NA		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

**If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am co-first author, along with Dr My VT Phan. Dr Phan set up the study at the sites and oversaw sample and data collection. I was responsible for all data cleaning, management and analysis. I corrected errors in the data from source documentation and generated the finalised dataset for analyses. Furthermore, I developed all of the STATA

	code for analysis and R code for figures. Dr Phan and I both generated the first draft of the manuscript. I created all final tables and figures and was the primary author involved in manuscript preparation and submission.

Student Signature: _____ **Date:** 16 Nov 2015

Supervisor Signature: _____ **Date:** 16/11/15

Corinne Thompson

From: Cathi Siegel <cbs15@case.edu>
Sent: Thursday, November 19, 2015 2:03 AM
To: Corinne Thompson
Subject: Re: Copyright for published manuscript

Yes, that's fine too, thanks.

On Tue, Nov 17, 2015 at 9:37 PM, Corinne Thompson <cthompson@oucru.org> wrote:
> Hi Cathi,
>
> Thanks very much for your reply. Can I further request permission to include the following article(s) that I published and authored with you:
>
> Thompson CN, Phan Vu Tra M, Nguyen Van Minh H, et al. A Prospective
> Multi-Center Observational Study of Children Hospitalized with
> Diarrhea in Ho Chi Minh City, Vietnam. Am. J. Trop. Med. Hyg. 2015;
> 95:1045–1052
>
> in the digital copy of my thesis which will be made publically available through LSHTM Research Online http://researchonline.lshtm.ac.uk/ . This is our institutional repository and is non commercial and openly available to all.
>
> Yours sincerely
>
> Corinne
>
> --
>
> Corinne Thompson, MSc
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> E-mail: cthompson@oucru.org
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>
>
> -----Original Message-----
> From: Cathi Siegel [mailto:cbs15@case.edu]
> Sent: Tuesday, November 17, 2015 10:19 PM
> To: Corinne Thompson
> Subject: Re: Copyright for published manuscript

>
> Yes, that's fine.
> Best regards,
> Cathi Siegel
>
>
> On Mon, Nov 16, 2015 at 9:43 PM, Corinne Thompson <cthompson@oucru.org> wrote:
>> Hi there,
>>
>>
>>
>> My name is Corinne Thompson and I am the first author on a manuscript
>> published earlier this year: doi:10.4269/ajtmh.14-0655
>>
>>
>>
>> I am a PhD student with the London School of Hygiene & Tropical
>> Medicine and I would like to include this published work in my thesis.
>> Would it be possible for AJTMH to please grant me permission to
>> include this manuscript in my PhD?
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>> Many thanks,
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>> Corinne
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>> Enteric Infections Group
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doi:10.4269/ajtmh.14-0655

A Prospective Multi-Center Observational Study of Children Hospitalized with Diarrhea in Ho Chi Minh City, Vietnam

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The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam; Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, United Kingdom; The London School of Hygiene and Tropical Medicine, London, United Kingdom; The Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom; Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, United Kingdom; The Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan; Gastrointestinal Bacteria Reference Unit, Public Health England, London, United Kingdom; Children's Hospital 1, Ho Chi Minh City, Vietnam; Children's Hospital 2, Ho Chi Minh City, Vietnam; Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam

Abstract. We performed a prospective multicenter study to address the lack of data on the etiology, clinical and demographic features of hospitalized pediatric diarrhea in Ho Chi Minh City (HCMC), Vietnam. Over 2,000 (1,419 symptomatic and 609 non-diarrheal control) children were enrolled in three hospitals over a 1-year period in 2009–2010. Aiming to detect a panel of pathogens, we identified a known diarrheal pathogen in stool samples from 1,067/1,419 (75.2%) children with diarrhea and from 81/609 (13.3%) children without diarrhea. Rotavirus predominated in the symptomatic children (664/1,419; 46.8%), followed by norovirus (293/1,419; 20.6%). The bacterial pathogens *Salmonella*, *Campylobacter*, and *Shigella* were cumulatively isolated from 204/1,419 (14.4%) diarrheal children and exhibited extensive antimicrobial resistance, most notably to fluoroquinolones and third-generation cephalosporins. We suggest renewed efforts in generation and implementation of policies to control the sale and prescription of antimicrobials to curb bacterial resistance and advise consideration of a subsidized rotavirus vaccination policy to limit the morbidity due to diarrheal disease in Vietnam.

INTRODUCTION

Childhood diarrhea remains a serious global public health issue, with an estimated 1.7 billion infections and 0.7 million deaths in children under 5 years annually, most of which occur in industrializing regions.[1,2] Rotavirus (RoV) and norovirus (NoV) are together responsible for an estimated 40% of severe diarrhea in children in low- and middle-income countries,[3] with bacterial pathogens *Shigella* spp., *Campylobacter* spp., and *Salmonella* spp. commonly identified as well.[4] *Cryptosporidium*, a protozoan, has also recently been found to cause a significant proportion of moderate to severe diarrhea in children < 5 years of age in resource-poor countries.[4] Diagnosis and treatment of diarrheal disease in such settings, however, is hampered by a lack of laboratory capacity, lack of specific clinical indicators, overuse of antimicrobial therapy, and a resultant increase in antimicrobial resistance.[5]

Diarrhea is a significant cause of morbidity in children in Ho Chi Minh City (HCMC), Vietnam,[6,7] yet there is limited data on etiology, clinical features, and prevalence of antimicrobial resistance among children hospitalized with diarrhea in this setting. HCMC is a densely populated, rapidly industrializing city that is home to over 8 million people in southern Vietnam.[8] Rampant antimicrobial usage in the community has led to alarming reports of third-generation cephalosporin and fluoroquinolone resistance among pathogenic and commensal gastrointestinal bacteria.[9,10] Furthermore, appropriate treatment and prevention strategies may be hindered by a lack of distinguishing, pathogen-specific clinical characteristics. To address gaps in knowledge regarding hospitalized pediatric diarrhea in HCMC, we conducted a cross-sectional hospital-based study aiming to describe etiological and clinical features, epidemiological characteristics, and antimicrobial susceptibility profiles of pediatric diarrheal disease in this rapidly developing southeast Asian city.

MATERIALS AND METHODS

Study sites and ethical approval. This prospective, hospital-based study was conducted at three hospitals in HCMC: Children's Hospital 1 (CH1), Children's Hospital 2 (CH2), and the Hospital for Tropical Diseases (HTD). CH1 and CH2 are the largest pediatric hospitals (1,500 beds each) in HCMC while HTD is a 500-bed referral hospital in southern Vietnam. The Scientific and Ethics Committee of CH1, CH2, HTD and the University of Oxford Tropical Research Ethics Committee (OxTREC No. 0109) approved this study. Written informed consent was required from parents or legal guardians prior to participation in the study.

Enrollment procedures and inclusion/exclusion criteria. Children ≤ 60 months of age who were admitted with acute diarrheal disease to the gastrointestinal wards of the three hospitals from May 2009 to April 2010 were considered for inclusion in this study as diarrheal cases. Inclusion criteria were diarrhea as the primary reason for admission (defined as three or more loose stools or at least one bloody loose stool within a 24-hour period, according to the World Health Organization [WHO] guidelines),[11] resident within the districts of HCMC, and no antimicrobial treatment within 3 days prior to study enrollment. We excluded children with a history of antimicrobial treatment in an effort to conserve resources as we

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†These authors contributed equally to this work.

rarely isolate any organisms from such patients in this setting (James Campbell, unpublished data). There were no additional exclusion criteria. The first five patients that met the inclusion criteria were enrolled at each study site on weekdays because of resource and staff limitations.

Children of the same age range (0–60 months) attending CH1 or CH2 for a health check or for other gastrointestinal issues unrelated to diarrhea, gastroenteritis, or other infectious diseases between March and December 2010 were invited for enrollment as hospital-based, non-diarrheal controls. Inclusion criteria included living within the districts of HCMC, no antimicrobial use within 3 days prior to hospital admission, and no history of diarrhea or respiratory illness within 7 days of study enrollment. There were no additional exclusion criteria.

Sample collection and microbiological methods. A stool specimen was collected in a sterile container from each enrollee as soon as possible and prior to any prescribed antimicrobial treatment. A specimen was collected within 24 hours of hospital admission to limit detection of nosocomial infection. Classical microbiological culturing was performed on all collected fresh stool samples on the day of sampling to isolate common diarrheal bacteria including *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp., *Plesiomonas* spp., and *Aeromonas* spp. as described previously.[12] Specific serotypes of *Shigella* spp. and *Salmonella* spp. were identified by slide agglutination with antigen grouping sera and monovalent antisera, and *Campylobacter jejuni* was differentiated from *Campylobacter coli* by the hippurate hydrolysis test as previously described.[12] A fresh smear of fecal specimen was prepared in phosphate buffered saline to examine the presence of *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* cysts.[12]

Antimicrobial susceptibility and extended-spectrum β-lactamase testing. The minimum inhibitory concentrations (MICs) of bacterial isolates were determined by E-test (AB Biodisk, Solna, Sweden) using the disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[13] Twelve antimicrobials were tested: ciprofloxacin (CIP), ceftriaxone (CRO), ceftazidime (CAZ), amoxicillin-clavulanic acid (AUG), erythromycin (ERY), ofloxacin (OFX), ampicillin (AMP), trimethoprim-sulfamethoxazole (SXT), azithromycin (AZT), chloramphenicol (CHL), gatifloxacin (GA), and nalidixic acid (NA). The production of extended-spectrum β-lactamases (ESBL) was detected using the double-disc synergy test: isolates with an increase in diameter of inhibitory zone of ≥ 5 mm because of the synergy of clavulanate were considered to be ESBL positive.[14]

Molecular detection of RoV and NoV. For molecular testing, total viral RNA was extracted, reverse transcribed into cDNA as previously described,[15] and used to detect RoV and NoV by reverse transcriptase polymerase chain reaction (RT-PCR). RoV detection was performed targeting the outer capsid genes (VP7 and VP4),[16] while NoV genogroup I (GI) and II (GII) were identified in separate PCR reactions targeting the conserved region overlapping open reading frame (ORF) 1–2.[17] All PCR amplicons were visualized on 2% agarose gels under ultraviolet (UV) light after staining with 3% ethidium bromide.

Molecular detection of pathogenic *Escherichia coli*. A total of 360 stool samples (210 from symptomatic cases and 150 from non-diarrheal controls) that were parasite negative and culture negative for all tested bacteria and RT-PCR negative for RoV and NoV were randomly selected to screen for the presence of pathogenic *E. coli* variants. Total nucleic acid was extracted from these samples using an automated MagNA Pure 96 nucleic extraction system (Roche Applied Sciences, West Sussex, United Kingdom) according to the manufacturer's recommendations. Multiple PCR reactions were performed directly on the extracted DNA to detect enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC). Multiplex real-time PCRs were performed to detect EHEC and EPEC in one duplex reaction[18] and to detect EIEC and EAEC in an additional duplex. ETEC was detected and classified as ETEC-LT (heat labile), ETEC-ST (heat stable), or ETEC-LT/ST using independent conventional PCR under previously described conditions.[19,20] PCR amplicons were visualized on 2% agarose gels under UV light after staining with 3% ethidium bromide.

Clinical and demographic data. A simple case report form (CRF) was completed for each enrolled patient by study clinicians to obtain data regarding symptoms, disease duration, and treatment regimens as per the standard care at the study hospitals. Study staff administered a confidential questionnaire detailing demographic, socioeconomic, and behavioral characteristics to all enrolled individuals. Average rainfall and temperature data for HCMC were obtained from the Vietnam Southern Regional Hydro-Meteorological Station. The nutritional status of all enrolled children was expressed as the weight-for-age Z (WAZ) score based on WHO growth standards[21]; children with a WAZ score value below –2 were considered malnourished.[22]

Statistical analyses. Tabulations of demographic, clinical, and laboratory characteristics among cases and non-diarrheal controls were performed with STATA 9.2 (StataCorp, College Station, TX) and compared using the χ^2 test, Fisher's exact test, or Mann–Whitney *U* test as appropriate. Two-sided *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Demographic characteristics. Over the study period, 1,419 diarrheal cases (referred hereafter as cases) and 609 non-diarrheal control children (referred hereafter as non-diarrheal controls) were enrolled; the demographic characteristics of the cases and non-diarrheal controls are shown in Table 1. Both the cases and non-diarrheal controls were more frequently male (64% and 53%, respectively) and had a combined median age of 12 months (interquartile range [IQR]: 8–20 months). The non-diarrheal controls were more likely to have a poor WAZ score than cases (13% versus 7%; $P < 0.001$, χ^2 test). Of cases and the non-diarrheal controls, 70% were frequently breast-fed as infants, yet the regular use of milk formula and probiotics was more common among the non-diarrheal controls (82%; 498/609 and 64%; 304/473, respectively) than the cases (58%; 819/1,419 and 14%; 121/861, respectively) ($P < 0.001$ for each comparison, χ^2 test). The majority of families of the enrollees (> 80%) resided within the urban districts of HCMC, as opposed to the peri-urban/rural areas. In addition, cases were more likely to report a lower income than controls. The households of more than half of all enrolees used a government pipeline as their major household water source; there were no significant differences in water source between cases and the diarrheal controls.

TABLE 1
The demographic characteristics of diarrheal and non-diarrheal children

Characteristic	Cases *n* (%) *N* = 1,419	Non-diarrheal controls *n* (%) *N* = 609	*P* value§
Male sex	905 (63.8)	322 (52.9)	< **0.001**
Median age (IQR) months	13 (8–19)	12 (8–20)	0.711
Poor WAZ*	93 (6.6)	76 (12.5)	< **0.001**
Breast-fed	1,017 (71.7)	465 (76.4)	**0.029**
Day care/nursery school attendance	223 (15.9)	93 (15.4)	0.837
Median household size (IQR)	6.5 (2–31)	6.4 (3–26)	0.445
Income bracket (monthly)			
< $145	422 (29.7)	136 (22.3)	< **0.001**
$145–242	532 (37.5)	211 (34.6)	
$243–483	326 (23)	168 (27.6)	
$484–725	90 (6.3)	61 (10.1)	
> $725	49 (3.5)	33 (5.4)	
Household water source			
Government pipeline	835 (59.0)	359 (59.0)	0.471
Well	501 (35.4)	223 (36.6)	
Other†	81 (5.7)	27 (4.4)	
Residential location‡			
Rural/peri-urban	261 (18.4)	76 (12.5)	**0.001**
Urban	1,158 (81.6)	533 (87.5)	

IQR = interquartile range; WAZ = weight-for-age Z score.
*Weight-for-age Z-score < –2.[21,22]
†Other household water sources include rainwater, well water, and water bought from governmental truck dispenser.
‡Rural/peri-urban and urban districts.
§*P* values through χ^2 or Mann–Whitney *U* test as appropriate.

The prevalence of enteric pathogens. At least one known enteric pathogen was identified in 75.2% (1,067/1,419) of stool samples from the cases and in 13.3% (81/609) of stool samples from the non-diarrheal controls (Table 2). The majority of enrollees with an isolate-positive stool sample (cases 91%; 970/1,067 and non-diarrheal controls 94%; 76/81) were infected with a single pathogen from the screened panel (see Methods). Of cases, 97 (9%) were infected with two pathogens; including combinations of bacteria (0.4%; *N* = 5), viruses (2.3%; *N* = 32), or virus and bacteria (4.2%; *N* = 60). RoV and NoV were identified in 46.8% (664/1,419) and 20.6% (293/1,419) of all cases, respectively (Table 2). The bacterial genera *Salmonella*, *Shigella*, and *Campylobacter* were isolated from 57 (4.0%), 48 (3.4%), and 31 (2.2%) cases, respectively. Of patients with an isolated bacterial pathogen, 67 (33%) cases had an additional pathogen (Table 2). No *Cryptosporidium* isolates were identified.

TABLE 2
Enteric pathogens identified in the stools of diarrheal cases and non-diarrheal controls

Organism	Cases *n* (%) *N* = 1,419	Non-diarrheal controls *n* (%) *N* = 609	*P* value*
Norovirus	241 (17.0)	15 (2.5)	< **0.001**
Rotavirus	590 (41.6)	10 (1.6)	< **0.001**
Campylobacter	31 (2.2)	16 (2.6)	0.544
jejuni	19 (1.3)	11 (1.8)	0.424
coli	12 (0.8)	5 (0.8)	0.955
Salmonella	57 (4.0)	34 (5.6)	0.118
Group B	35 (2.5)	12 (2.0)	0.496
Group C	8 (0.6)	0 (0.0)	0.115
Group D	4 (0.3)	1 (0.2)	1.000
spp.	9 (0.6)	21 (3.4)	< **0.001**
arizonae	1 (0.1)	0 (0)	1.000
Shigella	48 (3.4)	0 (0)	< **0.001**
flexneri	4 (0.3)	0 (0)	0.323
sonnei	44 (3.1)	0 (0)	< **0.001**
Other bacteria	2 (0.1)	1 (0.2)	1.000
Parasites	1 (0.1)	0 (0)	1.000
Mixed viral RoV/NoV	32 (2.3)	1 (0.2)	< **0.001**
Mixed viral bacterial	60 (4.2)	3 (0.5)	< **0.001**
Mixed bacteria	5 (0.4)	1 (0.2)	0.675
Total	1,067 (75.2)	81 (13.3)	< **0.001**

*P value from χ^2 test or Fisher's exact test, as appropriate; boldface indicates statistical significance.

In contrast to the dominance of viral infections among cases, bacterial pathogens were identified in a greater proportion than viruses among the non-diarrheal controls. *Salmonella* was the most commonly identified bacterial pathogen of the 609 non-diarrheal controls, isolated from a total of 39 (6.4%) stool samples (Table 2), while *Campylobacter* was identified in 16 (2.6%). None of the non-diarrheal controls were culture positive for *Shigella* spp. In addition, NoV and RoV were isolated from 17/609 (2.8%) and 13/609 (2.1%), respectively, non-diarrheal controls.

Pathogenic *E. coli* were detected by PCR amplification in 34% (72/210) and 55% (82/150) subset of randomly selected stool samples from the cases and non-diarrheal controls, respectively, in which no other pathogen was identified. As shown in Table 3, EPEC was the most common pathogenic *E. coli* variant detected, found more frequently in non-diarrheal controls (51%; 76/150) than in diarrheal children (19%; 39/210). However, there was significantly more atypical EPEC detected in non-diarrheal controls than in diarrheal cases (50% versus 18%; $P < 0.001$, χ^2 test). EAEC was the second most common *E. coli* variant detected, identified in the stools of a similar proportion of cases (9%; 18/210) and non-diarrheal controls (11%; 17/150). EIEC and ETEC were identified less frequently, but both were more commonly isolated in children with diarrhea as shown in Table 3.

Clinical manifestations. The clinical characteristics, type of diarrheal stool, and the presence of red and white blood cells in stool (by microscopy) were recorded for all diarrheal cases on admission (Table 4). Loose watery diarrhea was the most commonly recorded stool type (79%; 1,118/1,419), which was most prevalent among children who had a viral enteric pathogen in their stool (89%; 766/863). Approximately half of the cases infected with *Shigella* (49%; 23/47), *Campylobacter* (58%; 18/31), and *Salmonella* (49%; 28/57) presented with

TABLE 3
The prevalence of pathogenic *Escherichia coli* variants in the stools of diarrheal cases and non-diarrheal controls

E. coli variant	Cases *n* (%) *N* = 210	Non-diarrheal controls *n* (%) *N* = 150	*P* value*
EPEC	39 (18.6)	76 (50.7)	< **0.001**
Typical	1 (0.5)	1 (0.7)	1.000
Atypical	38 (18.1)	75 (50.0)	< **0.001**
EHEC	2 (1.0)	1 (0.7)	1.000
EAEC	18 (8.6)	17 (11.3)	0.471
EIEC	21 (10.0)	3 (2.0)	**0.002**
ETEC	12 (5.7)	2 (1.3)	**0.050**
LT	10 (4.8)	1 (0.7)	**0.029**
ST	1 (0.5)	1 (0.6)	1.000
LT and ST	1 (0.5)	0 (0.0)	1.000
Mixed infections	23 (11.0)	19 (12.6)	
Total	72 (34.3)	82 (54.7)	< **0.001**

EAEC = enteroaggregative; EHEC = enterohemorrhagic; EIEC = enteroinvasive; EPEC = enteropathogenic; ETEC = enterotoxigenic; LT = heat labile; ST = heat stable.
*P value from χ^2 test or Fisher's exact test, as appropriate; boldface indicates statistical significance.

TABLE 4
The clinical manifestations of viral- and bacterial-associated diarrhea

Characteristic	Viral infection *n* (% or IQR) *N* = 863	Bacterial infection *n* (% or IQR) *N* = 143	Mixed viral/bacterial infection *n* (% or IQR) *N* = 60	*P* value‖
Bloody diarrhea	4 (0.5)	12 (8.4)	0 (0)	< **0.001**
Mucoid diarrhea	93 (10.8)	60 (42.0)	16 (26.7)	< **0.001**
Watery diarrhea	766 (86.1)	71 (49.7)	44 (73.3)	< **0.001**
Mild fever	480 (55.6)	73 (51.0)	33 (55.0)	0.319
Severe fever	165 (19.1)	45 (31.5)	4 (6.7)	**0.001**
Dehydration	92 (10.7)	4 (2.8)	6 (10.0)	**0.002**
Vomiting	730 (84.6)	89 (62.2)	46 (76.7)	< **0.001**
Cough	274 (31.7)	38 (26.6)	20 (33.3)	0.242
Abdominal pain	40 (4.6)	30 (21.0)	3 (5.0)	< **0.001**
Anorexia	533 (61.8)	74 (51.7)	38 (63.3)	**0.027**
WBC + stool	191 (22.1)	100 (69.9)	27 (45.0)	< **0.001**
RBC + stool	94 (10.9)	78 (54.5)	16 (26.7)	< **0.001**
Average daily episodes*	4 (3–7)	5 (3–8)	5.5 (3–8.5)	0.414
Maximum daily episodes†	10 (6–13)	10 (6–13)	10 (6–12)	0.904
Length of illness‡	2 (2–3)	2 (1–3)	2 (1–3)	< **0.001**
Length of stay§	5 (3–7)	4 (3–7)	5 (2.5–7)	0.5247

RBC = red blood cells; WBC = white blood cells.
*Average number of diarrheal episodes in a 24-hour period, as reported by the parent/guardian.
†Maximum number of diarrheal episodes in a 24-hour period, as reported by the parent/guardian.
‡Prior to hospitalization (days).
§Hospitalization duration (days).
‖Comparison of viral and bacterial infections only. Fisher's exact, χ^2 or Mann–Whitney *U* test as appropriate; boldface indicates statistical significance.

visible blood or mucus in their stool. The majority of all cases had moderate (37.2–39°C) (52%; 744/1,419) or high fever (> 39°C) (22%; 312/1,419) in addition to vomiting (78%; 1,100/1,419).

Symptomatic children with a viral pathogen in their stool were more likely to present with dehydration and vomiting and to have had diarrhea for a longer period prior to hospitalization, than those with a bacterial pathogen, as shown in Table 4. The symptomatic cases with a bacterial pathogen in their stool were more likely to present with abdominal pain, severe fever and to have blood cell-positive stool smears. The median age of cases was comparable when stratified by the pathogen(s) found in the stools, with the exception of children with a *Shigella* infection (median: 31 months; IQR: 20–36 months), who were significantly older than cases with other enteric pathogens identified in their stool (median: 13 months; IQR: 8–19 months) ($P < 0.001$, Mann–Whitney *U* test).

Diarrheal treatment regimes. Cases with a confirmed bacterial infection were more likely to be prescribed an antimicrobial on presentation (74%; 106/143) than those with a viral infection (38%; 324/862) ($P < 0.001$, χ^2 test), where the prescription of antimicrobials was determined by clinical presentation before etiological investigation. Approximately half (48%; 29/60) of those with a combined bacterial/viral infection were prescribed an antimicrobial, and 60% (210/352) of those with diarrhea of unknown origin were treated with antimicrobials. Children with visible blood and/or mucus in their stool were prescribed antimicrobials frequently (75%; 226/301) as were children with high fever (68%; 214/313). The most commonly prescribed groups of antimicrobials were fluoroquinolones (61%; 438/714) and third-generation cephalosporins (22%; 160/714). Zinc and probiotics were prescribed slightly more frequently to those who had a confirmed viral infection (74%; 635/862 and 69%; 591/862, respectively) than those who had a bacterial infection (66%; 95/143 and 62%; 88/143, respectively).

Spatial and temporal distribution of enteric pathogens. Using the available location data from the diarrheal patients, we found no association between the relative proportion of each pathogen and population density or urban/peri-urban locations, with RoV and NoV predominating in all districts of the city. In addition, we found no association between the frequency of cases caused by any of the identified pathogens and monthly mean temperature or rainfall, with the exception of a positive correlation between rainfall and the isolation of *Shigella* spp. (Spearman's correlation coefficient [r] = 0.592, $P = 0.043$). RoV was identified more frequently in the drier months (January–March), although the relationship was not significant ($r = -0.417$, $P = 0.178$) (Figure 1).

Antimicrobial susceptibility. The MIC distribution of the *Campylobacter*, *Shigella*, and *Salmonella* isolated from the cases against selected antimicrobials are shown in Table 5. The majority (≥ 80%) of the *Campylobacter* isolates exhibited resistance to NA and CIP, with only 8% (5/64) resistant to ERY. A large proportion (≥ 75%) of the 62 *Shigella* isolates were also resistant to NA and CRO. However, the *Salmonella* isolates were comparatively more susceptible to both fluoroquinolones and third-generation cephalosporins, with few (≤ 20%) isolates displaying resistance to CRO, CIP, or NA. Notably, the *Campylobacter* and *Shigella* isolates were relatively susceptible to CHL, with resistance identified in ≤ 2% of *Campylobacter* isolates, and in ≤ 7% of *Shigella* isolates. Many (40%) of the *Salmonella* isolates exhibited resistance to CHL. The majority (≥ 90%) of the *Campylobacter* and *Shigella* isolates demonstrated resistance to ≥ 3 classes of antimicrobials, and a high proportion of the *Shigella* isolates were ESBL positive (75%; 47/62). In contrast, a

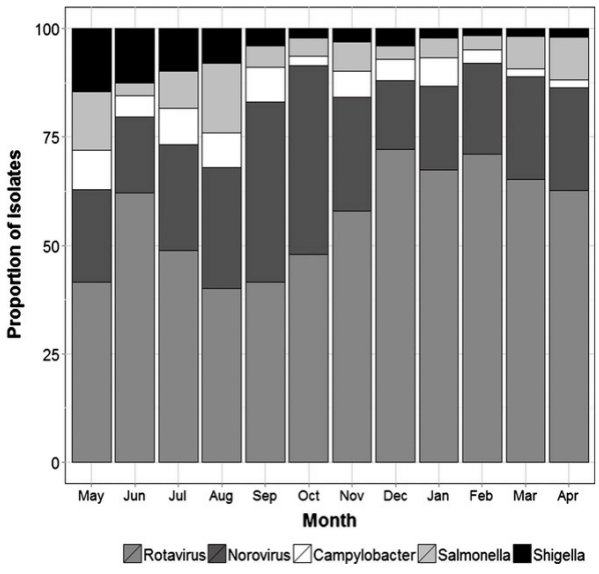

FIGURE 1. Proportion of various diarrheal etiologies among total isolates identified per month over the course of the study period. The proportion of isolates identified as rotavirus (medium gray), norovirus (dark gray), *Campylobacter* spp. (white), *Salmonella* spp. (light gray), and *Shigella* spp. (black) identified over each month of the study period (May 2009–April 2010) are shown as a stacked bar chart.

TABLE 5
Antimicrobial resistance among *Campylobacter*, *Salmonella*, and *Shigella* from diarrheal cases

	Campylobacter				*Salmonella*						*Shigella*		
Antimicrobial	Total (%)	*coli* (%)	*jejuni* (%)	Spp. (%)	Total (%)	Gp B (%)	Gp C (%)	Gp D (%)	Spp. (%)	*ariz* (%)	Total (%)	*flexneri* (%)	*sonnei* (%)
AMP	17/66 (26.3)	5/20 (28)	12/44 (26.5)	0/2 (0)	48/78 (48.1)	36/46 (65.5)	3/10 (27.2)	4/5 (80)	4/15 (18.7)	1/2 (50)	48/62 (77.4)	3/4 (75)	45/58 (77.5)
AMC	2/66 (3)	0/20 (0)	2/44 (4.5)	0/2 (0)	1/76 (1.3)	1/44 (2.3)	0/10 (0)	0/5 (0)	0/15 (0)	0/2 (0)	1/60 (1.7)	0/4 (0)	1/56 (1.8)
CAZ	10/66 (15.1)	5/20 (25)	5/44 (11.3)	0/2 (0)	6/78 (7.7)	5/46 (10.8)	1/10 (10)	0/5 (0)	0/15 (0)	0/2 (0)	1/62 (1.6)	0/4 (0)	1/58 (1.7)
CIP	52/65 (80.0)	20/20 (100)	30/43 (69.7)	0/2 (0)	4/76 (5.3)	4/44 (9.1)	0/10 (0)	0/5 (0)	0/15 (0)	0/2 (0)	1/61 (1.6)	0/4 (0)	1/57 (1.8)
GAT	8/66 (12.1)	2/20 (10.0)	6/44 (13.6)	0/2 (0)	0/78 (0)	0/46 (0)	0/10 (0)	0/5 (0)	0/15 (0)	0/2 (0)	0/62 (0)	0/4 (0)	0/58 (0)
OFL	54/66 (81.8)	20/20 (100)	32/44 (72.7)	2/2 (100)	3/78 (3.8)	2/46 (4.3)	0/10 (0)	1/5 (20)	1/15 (6.7)	0/2 (0)	1/62 (1.6)	0/4 (0)	1/58 (1.7)
CHL	1/66 (1.5)	0/20 (0)	1/44 (2.3)	0/2 (0)	30/77 (38.9)	24/45 (53.3)	2/10 (20)	2/5 (40)	2/15 (13.3)	0/2 (0)	4/61 (6.6)	3/4 (75)	1/57 (1.8)
TMP	51/65 (78.4)	17/20 (85)	32/43 (74.4)	2/2 (100)	30/78 (38.4)	24/46 (52.1)	2/10 (20)	2/5 (40)	2/15 (13.3)	0/2 (0)	55/59 (93.2)	3/4 (75)	52/55 (94.5)
NA	56/66 (84.8)	20/20 (100)	34/44 (77.2)	2/2 (100)	14/78 (17.9)	11/46 (23.9)	0/10 (0)	2/5 (40)	1/15 (6.7)	0/2 (0)	56/62 (90.3)	2/4 (50)	54/58 (93.1)
ERY	5/64 (7.8)	5/20 (25.0)	0/42 (0)	0/2 (0)	–	–	–	0/5 (0)	1/15 (6.7)	0/2 (0)	–	–	–
CRO	–	–	–	–	10/78 (12.8)	8/46 (17.3)	1/10 (10)	0/5 (0)	1/15 (6.7)	0/2 (0)	45/62 (72.5)	0/4 (0)	45/58 (77.5)

AMC = augmentin; AMP = ampicillin; CAZ = ceftazidime; CHL = chloramphenicol; CIP = ciprofloxacin; CRO = ceftriaxone; ERY = erythromycin; GAT = gatifloxacin; NA = nalidixic acid; OFL = ofloxacin; TMP = trimethoprim.

negligible proportion of *Salmonella* isolates exhibited ESBL activity (3%; 2/78).

In general, a lower proportion of pathogens isolated from stools of the non-diarrheal controls demonstrated antimicrobial resistance compared with isolates from symptomatic cases. Specifically, all *Salmonella* isolates from the non-diarrheal controls were susceptible to CIP, CRO, and CAZ. Resistance to CHL was low (2.6%; 1/39). However, many of the *Campylobacter* isolates from the non-diarrheal control stool samples were resistant to CIP (68.7%; 11/16), NA (62.5%; 10/16), and CHL (18.7%; 3/16).

DISCUSSION

This is one of the few studies addressing the causative agents of diarrheal disease in southern Vietnam. Using the described methods, we were able to detect at least one known diarrheal pathogen in 75% of all diarrheal cases, which is comparable to the findings by previous case–control studies conducted in the north of Vietnam (67%; $N = 587$),[23] and in seven developing countries in Africa and Asia within The Global Enteric Multicenter Study (GEMS) (83%; $N = 9{,}439$).[4] In this study, the remaining 25% cases in which we could not isolate a pathogen may correspond with disease caused by alternative nontargeted pathogens, limited diagnostic sensitivity, unreported pretreatment with antimicrobials, or other causes, such as food allergy, malabsorption, or maldigestion.[24]

Diarrheal disease in young children in industrialized countries is generally considered to be caused by viral pathogens, while bacterial and parasitic diarrheal agents are generally considered more prevalent in industrializing countries.[25] Here, we identified viral pathogens more frequently than bacterial pathogens in diarrheal cases, potentially reflecting the effect of the recent economic transition in Vietnam on the epidemiology of enteric pathogens in HCMC.[7] Furthermore, the large proportion of children hospitalized with RoV-induced diarrhea in this setting predicts that uptake of RoV vaccine would have a considerable impact on the diarrheal disease burden in this population. In Vietnam, the current cost of RoV immunization is prohibitive (approximately $75 for a full course of RotaTeq [Merck, West Point, PA] or RotaRix [GSK, Middlesex, UK]) and neither of these licensed vaccines are currently included in Vietnam's Extended Program of Immunization (EPI) schedule. We therefore suggest that the integration of RoV vaccine into the EPI schedule should be considered as a matter of necessity.[26] An alternate way forward for RoV vaccination in Vietnam would be through regional mass production of a generic vaccine, such as the live-attenuated monovalent G1P[8] (Rotavin-M1, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam) vaccine that has recently undergone trials in Vietnam.[27,28] This approach may reduce the cost of RoV immunization to the Vietnamese health service, permitting a greater national coverage and thus greater impact.

The variable pathogenicity of *E. coli* pathovars in addition to the cost and technical difficulties in diagnostic detection of this group have greatly hindered the understanding of disease epidemiology and pathogenesis of this bacterial species in childhood diarrhea, particularly in resource-limited settings. We were able to detect the presence of five different *E. coli* pathogenic variants from a random cross section of stools from cases and non-diarrheal controls without an alternate

identified etiology by PCR amplification. We found that atypical EPEC was the most common variant in both children with and without diarrhea. Recent evidence from the GEMS study suggested that atypical EPEC was not associated with moderate-to-severe diarrhea,[4] confirmed by our findings as non-diarrheal controls were more likely to be infected with this variant. This association with EPEC has been previously observed.[29] EPEC has also been reported to be associated with a more persistent clinical diarrheal syndrome rather than the acute diarrheal syndrome that was assessed here.[30] We additionally found that ETEC-LT was predominant and significantly associated with cases in comparison to non-diarrheal controls in this study. However, findings from GEMS suggest that ETEC-ST is a significant diarrheal pathogen and ETEC-LT is not a significant cause of moderate-to-severe diarrhea.[4] We conclude that this discrepancy reflects the differing epidemiology of ETEC between settings, which may be related to different routes of transmission, behavioral risk factors, and water quality.[31]

The rate of pathogen detection in our non-diarrheal control population was much lower than that identified in the GEMS study (13% versus 72%).[4] The GEMS study screened for a wider variety of pathogens, often using more sensitive assays, which could in part explain the difference. The exclusion of children who had received recent antimicrobial treatment may have biased our non-diarrheal control population as well. Also, the controls were more likely to report higher socioeconomic status indicators, and are thus likely from a different epidemiological population than the cases, which also may explain the low rate of pathogen detection in this group. However, one of the more notable findings from this study was that *Salmonella* spp. was detected in a similar proportion in cases and non-diarrheal controls (6%). *Campylobacter* spp., although more common in cases (5%), was also identified in 3% non-diarrheal controls. Recent case–control studies conducted in southeast Asia have found a high prevalence of *Campylobacter* spp. and *Salmonella* spp. from healthy non-diarrheal controls in rural Thailand and Cambodia.[32,33] These data imply that asymptomatic/transient infection with these organisms may be substantial in these areas; further research is warranted to determine the clinical relevance and role in disease transmission in the community.

We found remarkable levels of antimicrobial resistance in the pathogens isolated as part of this study; > 90% of *Campylobacter* spp. and *Shigella* and > 50% of *Salmonella* spp. isolates exhibited resistance to three or more classes of antimicrobials. Reduced susceptibility and resistance to broad-spectrum antimicrobials in enteric pathogens is becoming increasingly reported across Asia, and our data support the notion that resistance to multiple antimicrobial groups is common across multiple genera of enteric pathogens.[34] For example, we found an exceptionally high prevalence of ciprofloxacin-resistant *Campylobacter*, which has been recorded in several Asian countries, including Cambodia, India, and China.[33,35,36] Antimicrobial therapy is not generally recommended to treat non-dysenteric diarrhea, although ciprofloxacin is commonly used as a first-line antimicrobial in the case of profuse or bloody mucoid diarrhea.[37] As routine identification of any causative agents of diarrhea is not performed in hospitals in Vietnam, patients are treated following standard Vietnamese treatment guidelines (ciprofloxacin or ceftriaxone as first- and second-line therapies) based on clinical observations, which may be insensitive in distinguishing viral and bacterial infections. Developing affordable rapid point-of-care diagnosis should be considered to help clinicians choose more appropriate antimicrobial regimens for diarrheal patients in addition to encouraging continued use of rehydration and zinc supplementation.

Ceftriaxone, a third-generation cephalosporin, is recommended by the WHO as an alternative treatment of severe infectious diarrhea and shigellosis.[11,37] Yet the prevalence of ceftriaxone resistance has increased markedly, particularly in *Shigella* spp., in this location in the past decade.[7,10] The first ESBL-mediated ceftriaxone-resistant *Shigella* in southern Vietnam was isolated from a pediatric diarrheal patient in 2001 at the HTD, HCMC.[10] The prevalence of ceftriaxone-resistant *Shigella* strains found in this study (75%) was triple that of the 2007–2008 period in pediatric diarrheal patients in the same hospital (23%; $N = 103$).[7] The true prevalence of these resistant *Shigella* strains circulating in the community may be vastly underestimated because of a lack of routine diagnosis and antimicrobial resistance surveillance in the region. Moreover, we postulate that antimicrobial-resistant bacteria are circulating in commensal enteric microbiota in the community at high levels.[9] This hypothesis, in addition to the high reported rates of antimicrobial usage and resistance in poultry in Vietnam,[38] would increase the likelihood of antimicrobial resistance gene transfer, thus increasing the rate of emergence of multidrug-resistant strains and limiting effective therapeutic regimes for treating patients with severe or life-threatening bacterial infections.[10] Developing solutions against antimicrobial resistance is becoming a serious global challenge as we enter the "postantibiotic era."[39,40] In this context, we reiterate that regulating antimicrobial usage in the community and agriculture as well as improved control of hospital prescription practices may be a critically necessary strategy of slowing the rate of increasing antimicrobial resistance in this location.

Our study has some limitations. First, our non-diarrheal control population may not adequately represent all children without diarrhea in this community. The majority of the non-diarrheal controls were children attending a routine clinic for health checks, which may result in the introduction of biases. Second, we included only the first five patients seen each day; hence the description of seasonal patterns is limited by the recruitment pattern. Next, the proportion of pathogen-positive stool samples may be underestimated because our diagnostic methods targeted a limited group of pathogens, potentially missing other enteric pathogens such as adenovirus, astrovirus, and helminths. In addition, viral etiologies were likely identified more often due to sensitivity differences between PCR-based diagnostics compared with culture techniques used for bacterial pathogens and due to the exclusion of patients who had received recent antimicrobial therapy. The lack of *Cryptosporidium* isolates specifically, given the high prevalence identified in the GEMS study,[4] is likely due to the poor sensitivity of microscopy.[41] Finally, our study was hospital based, thus our passive enrollment and case detection was entirely dependent on health-care-seeking behavior. Therefore, our study will inevitably skew results toward the moderate-to-severe end of the disease spectrum, as the bulk of mild infections remain undetected in the community. Notwithstanding these limitations, we suggest that our clinical observations, etiology, and prevalence data are informative

and likely to be broadly representative of hospitalized diarrhea in this setting and other economically transitioning regions in southeast Asia.

In conclusion, we identified a known enteric pathogen in 75% cases of hospitalized pediatric diarrhea in HCMC, with RoV and NoV being the most frequently identified. While bacterial pathogens were identified in only 14% cases, alarming rates of antimicrobial resistance to recommended first- and second-line therapies are likely to result in a growing burden of hospitalized diarrhea in young children in this setting. We suggest renewed efforts in generation and implementation of policies to control the sale and prescription of antimicrobials to curb bacterial resistance and advise consideration of a subsidized RoV vaccination policy to limit the morbidity due to diarrheal disease in Vietnam.

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4 RESEARCH PAPER 2: The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam

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Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Health and Place		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am co-first author, along with Dr Jon Zelner of Columbia University. I collated and cleaned all of the hospital data (>500,000 records) including overseeing the extensive cleaning of the geographic data. I also was responsible for securing the climate data from the Vietnamese government. I performed all of the geostatistical mapping analyses. Dr

	Zelner developed the R code for the mixed effects model and goodness of fit evaluations. I wrote the manuscript, prepared all figures and tables and was responsbile for the submission procedures.

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The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam

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ABSTRACT

It is predicted that the integration of climate-based early warning systems into existing action plans will facilitate the timely provision of interventions to diarrheal disease epidemics in resource-poor settings. Diarrhea remains a considerable public health problem in Ho Chi Minh City (HCMC), Vietnam and we aimed to quantify variation in the impact of environmental conditions on diarrheal disease risk across the city. Using all inpatient diarrheal admissions data from three large hospitals within HCMC, we developed a mixed effects regression model to differentiate district-level variation in risk due to environmental conditions from the overarching seasonality of diarrheal disease hospitalization in HCMC. We identified considerable spatial heterogeneity in the risk of all-cause diarrhea across districts of HCMC with low elevation and differential responses to flooding, air temperature, and humidity driving further spatial heterogeneity in diarrheal disease risk. The incorporation of these results into predictive forecasting algorithms will provide a powerful resource to aid diarrheal disease prevention and control practices in HCMC and other similar settings.

1. Introduction

Diarrheal disease is a leading cause of childhood morbidity and mortality worldwide, with an estimated 1.7 billion infections and 0.7 million deaths annually (Walker et al., 2013). Oral rehydration, antimicrobials, and intravenous fluids are proven to save lives in outbreaks, and prevention measures such as vaccination and improved water, sanitation and hygiene (WASH) reduce endemic diarrheal disease incidence (Bhutta et al., 2013; Santosham et al., 2010). Nonetheless, the general uptake and sustainability of these measures globally is limited (Bhutta et al., 2013; Forsberg et al., 2007). Innovative techniques to address the massive burden of

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diarrheal disease are required. Akanda and colleagues recently proposed that climate-based surveillance should be factored into control strategies for diarrheal disease (Akanda et al., 2014). The authors argued that using climatic and remote sensing data can help predict diarrheal epidemics driven by environmental exposures in vulnerable communities up to three months prior to occurrence. This early warning mechanism allows time to preemptively administer an immunization program or provide WASH interventions such as water filtration devices (Akanda et al., 2012, 2014). Understanding the nature and magnitude of the relationships between diarrheal disease epidemiology and factors such as temperature, flooding and humidity should allow for the development of setting- or regionally-specific predictive models for climate-based early warning systems (Jutla et al., 2013).

We sought to describe the impact of environmental and climate conditions on the risk of diarrheal disease in Ho Chi Minh City (HCMC) in southern Vietnam. Considered an "Asian coastal megacity", HCMC is a rapidly industrializing low-lying city of 7.5 million people (The World Bank, 2010). Diarrheal disease remains a problem in Vietnam, with an estimated incidence of 271/1000 infant years in children under 12 months of age (Anders et al., 2015). Management of diarrhea in this resource-limited setting has become more challenging in recent years because of diminishing antimicrobial susceptibility in causative bacteria (Le et al., 2009; Vinh et al., 2009). Although seasonality of hospitalized diarrheal disease in this area has previously been recognized (Thompson et al., 2015), the effects of climate and weather on reported diarrhea risk have not been quantified.

In 2012 the UN reported that 98% and 93% of urban Vietnamese have access to improved drinking water sources and sanitation facilities, respectively (World Health Organization and UNICEF, 2014). However, rapid population growth, lack of investment and aging water and sewage infrastructure have led to alarmingly polluted waterways and expose residents of HCMC to considerable risk (Ha et al., 2008; Vo, 2007; Wust et al., 2002). The water of the Saigon and Dong Nai rivers is used as the drinking water for the population of HCMC, with limited purification or controls on industrial and domestic waste discharge (Hong et al., 2000; Vo, 2007). For example, it is estimated that only 40% of waste water from industrial and hospital sources discharged into the Saigon River is treated (Vo, 2007). Furthermore, poor residents who live along the canals and in settlements in HCMC often resort to groundwater use, which has been shown to be contaminated with human and animal excreta as well as toxic industrial waste (Ha et al., 2008; Hong et al., 2000).

Characterizing the role of environmental factors in diarrheal disease risk is challenging due to a general lack of pathogen-specific diagnoses, with "all-cause" diarrhea data reflecting a combination of viral, parasitic and bacterial pathogens, which vary in transmission dynamics and sensitivities to environmental conditions. Furthermore, seasonal patterns of diarrheal disease hospitalization may correlate with seasonal environmental factors even when no causal relationship exists; i.e. the transmissibility of the underlying pathogens may vary seasonally as a function of seasonal variation in contact behavior (Nichols et al., 2012; Pitzer et al., 2009), birthrates and other demographic factors (Cannon et al., 2009), or other unmeasured weather and climate factors (Kolstad and Johansson, 2011; Levy et al., 2009; Wu et al., 2013). To quantify the impact of weather and the environment on diarrheal risk in HCMC, we used a large, spatially explicit dataset of pediatric diarrheal admissions from three hospitals. We explored inherent seasonality of reported diarrhea at a city level and examined the sensitivity of each district to the effects of climate on risk to highlight localized drivers of diarrhea. Our work identifies marked spatial heterogeneity in risk of reported diarrhea in HCMC corresponding to differences in elevation and localized sensitivity to seasonal climate patterns; such results can be used to inform future climate-based predictive algorithms in this region for efficient targeting of public health and economic resources.

2. Methods

2.1. Patient data

To investigate the impact of local environmental factors on rates of diarrheal disease hospitalization across HCMC, data on all children <16 years of age, who were living in HCMC and admitted with diarrheal disease was collected from three large hospitals in HCMC for the period 2005–2010, inclusive. Data from Children's Hospital 1 (CH1) and Children's Hospital 2 (CH2) spanned the period from 2005 to 2010. Data from 2008 to 2010 was collected from the Hospital for Tropical Disease (HTD). For each patient, information on age, sex, date of admission, ward and district of residence, and ICD10 code on admission were provided. Any patient with an ICD10 diagnosis contained under the WHO-defined heading of "Intestinal infectious diseases" (codes A00-A09) (WHO, 2010) was classified as having diarrheal disease. A total of 443,295 children resident in HCMC under the age of 16 were admitted to CH1 and CH2 over the six-year period, of which 51,599 (11.6%) were admitted with diarrheal disease. HTD reported a total of 36,624 admissions from 2008 to 2010, of which 7174 (19.6%) were admitted with diarrheal disease.

2.2. Environmental and demographic data

Weather and climate data measured at the city level were obtained from the Ministry of Natural Resources and Environment of Vietnam (Loc, 2013). These include: citywide weekly average relative humidity (%), temperature (°C), rainfall (mm) and level of the Don Dien River of HCMC (cm above or below long-term average river level) during 2005–2010. To assess the relative impacts of seasonal variation in these environmental drivers, these covariates were standardized to have zero-mean and unit variance. The minimum and maximum values of the mean-standardized climate factors are as follows, (1) humidity: -2.7, 2.2; (2) temperature: -3.2, 3.1; (3) rainfall: -0.9, 4.5; (4) river level: -2.0, 2.3. Shuttle Radar Topography Mission (SRTM) elevation data was obtained from the CGIAR Consortium for Spatial Information (CGIAR-CSI) (Jarvis et al., 2008). Data on ward-specific populations were extracted from the 2009 Population and Housing Census (General Statistics Office of Vietnam, 2010). District-level populations were available at the beginning of 2005 and for each year from 2008 to 2010 from the 2011 Statistical Yearbook of HCMC (Statistical Office in Ho Chi Minh City, 2012). Weekly population sizes for each district were estimated from annual census estimates by linear interpolation (see Fig. S1).

2.3. Spatial information

There are 24 administrative districts in Ho Chi Minh City (HCMC) (Table S1) with a median area of 22 km^2, range (4–704 km^2) and median population density of 20,340/km^2 (range: 98–45,370/km^2). Additionally, within each district there are a series of wards that vary in size and number. There are a total of 322 wards within HCMC, with a median area of 1.2 km^2 (range: 0.1–122 km^2), and median population density 24,200/km^2 (range: 47–125,000/km^2).

Home addresses for all diarrheal disease inpatients were geocoded to district and ward centroids of HCMC. Spatially smoothed rates of hospitalization at the ward level were estimated using Empirical Bayesian Kriging (EBK) (Pfeiffer et al., 2008). EBK allows

estimation of spatially smoothed small-area rates by combining local information with weighted data from neighboring areas. Reported rate surface predictions and their associated variance (reflecting uncertainty in variogram estimation) were generated for the entirety of HCMC. More specifically, a new semivariogram is estimated for each of a set of overlapping subsets of the input data. These are then combined to obtain smoothed rates while accounting for variability in the strength of local spatial correlation across the map. EBK and mapping were performed using ArcGIS v10.2 (ESRI, California).

2.4. Multivariate model

We utilized a Poisson generalized linear mixed model (Gelman and Hill, 2006) to distinguish variation in reported diarrheal disease risk from seasonal epidemics and other city-level seasonal factors from district-level variation in response to climate factors. This model, with the outcome of monthly rate of diarrheal cases per population per district, included: (1) fixed-effects for district attributes (e.g. elevation), (2) monthly random intercepts to account for seasonal and district-level heterogeneity, and (3) district-level random intercepts and slopes to account for variation in response to city-level weather and climate factors. To ensure that our parameter estimates reflect per-capita rates, all models included an offset term for the logarithm of the population of district i at week t. Mixed effects analysis was performed in R (v3.02) using lme4 (v1.1–7) for R (Bates et al., 2014); plots were created using ggplot2 (v0.93) (Wickham, 2009). Goodness-of-fit analyses were performed and are described in the SI.

We included a fixed effect term to adjust for distance to the largest and most centrally located hospital (CH1) to ensure our estimates of district-level effects were not artifacts of differential reporting due to travel distance. All three hospitals are located in central HCMC, and inclusion of terms for distance to all hospitals instead of CH1 alone did not impact our results. We included lag terms for the district-level per-capita diarrheal hospitalization rate over the preceding eight weeks to account for temporal autocorrelation. Next, risks experienced simultaneously across all districts of HCMC were represented by random intercepts for each month from 2005 to 2010. The use of a monthly interval for these effects ensured that if a temporally varying district-level weather or climate effect was strongly correlated with citywide seasonal patterns, these district-level effects were still captured in the weekly data. In addition, the use of a monthly random intercept allowed us to capture short-term spatial lags in these effects.

Next, we included a random intercept for each district to account for baseline variation in district-level diarrheal hospitalization. We also included district-level random slope terms for standardized values of city-level climate variables. Changes beginning around 2008 to policies regarding health insurance for children under the age of six years may have impacted reporting in some locations around this time (specifically the catchment areas of CH1 and CH2) (Shieh et al., 2013). To account for changes in reporting following this policy shift, a district-level random slope term indicating which weeks occurred during and after 2008 was included in the model in addition to the district-level weather and climate random effects (Fig. S2). Population density was not associated with variation in district-level risk and was not included in the final model.

To assess the agreement between our model and the district-level data, we generated 1000 simulated datasets and visually compared the range of these simulated values to the weekly data. We also calculated deviance residuals to provide a quantitative measure of goodness-of-fit. The resulting district-level predictions (Fig. S5) and deviance (Fig. S6) displayed a generally good agreement between the fitted model and the source data. To ensure that district-level autocorrelation was adequately accounted for, we examined the partial autocorrelation function of the deviance residuals for each district up to a 10-week lag (Fig. S7). The partial autocorrelation function (PACF) represents the autocorrelation between two points t and $t+1$ in a series after adjustment for the previous $t-1$ terms, suggests that district-level temporal autocorrelation is adequately accounted for by our model. The resulting figures indicate good agreement between the model and data at the district level.

Ethical approval for this study was granted by all local hospital ethical committees and the Oxford Tropical Research Ethics Committee.

3. Results

3.1. Patterns of diarrheal disease reporting in Ho Chi Minh City

Of the 479,919 childhood admissions across three hospitals over 2005–2010, 58,773 (12.3%) were attributed to diarrheal disease. Only ICD10s of the acute respiratory infection syndrome (J00-J06) (WHO, 2010) accounted for more admissions (14%). The most commonly documented diarrheal ICD10 codes were A09 (Gastroenteritis [GE]/colitis of infectious origin):27%, A09.1 (diarrhea and GE of presumed infectious origin with mild dehydration):27%, A09.2 (diarrhea and GE of presumed infectious origin with severe dehydration):19% and A04.9 (bacterial intestinal infection):9%.

The median age of the hospitalized diarrheal cases in HCMC was 1.2 years (interquartile range: 0.7–2.1 years), and 63% of these cases were male.

A consistent pattern of seasonality in diarrhea hospitalizations was apparent from the citywide count data (Fig 1A). Throughout the sampled years the burden of diarrheal disease was highest during the drier months from January to March with a mean of 10% of yearly cases reported in each of these months (range: 7.5–12.4%). There was a corresponding trough in August–September, with a mean of 7% of annual cases reported during each month (range: 6.1–8.2%). Individual district-level time series varied in magnitude and variability of the yearly peak of reported cases in the dry season (Fig. S3). The overall rate of reported diarrhea across HCMC did not differ spatially when stratified by age of patients or by season (dry and wet).

The minimum district-level rate of hospitalized diarrhea in children <16 years of age was 11.9/100,000 population (range: 1.1–42.2/100,000). The smoothed ward-level rates in Fig. 2A illustrate substantial heterogeneity in overall risk of diarrheal disease across HCMC. We observed that wards situated adjacent to waterways in the central regions of the city were more likely to have a higher rate of all-cause diarrhea in comparison to the normalized citywide monthly mean. The minimum reported incidence rates in the waterway-laden areas (specifically in wards within district 6 and 8) exceeded 50 cases/100,000 population in some months. These central areas are amongst the most densely populated in the city with a mean population density of 44,300/km^2 (Fig. 2C), and are situated below the average city elevation (2.8 m) at a ward mean of 1.14 m above sea level (Fig. 2D). The trend of an elevated rate of diarrheal hospitalizations extended south along the river ways and canals through districts 7, 13 and 20 (district locations shown in Fig. 5).

3.2. Bivariate analysis

River level, humidity, rainfall and temperature varied seasonally in all study years (Fig. 1B). Using bivariate Poisson regression we found that river level correlated positively with citywide per-capita diarrheal disease rates (Relative Risk (RR):1.07, 95%

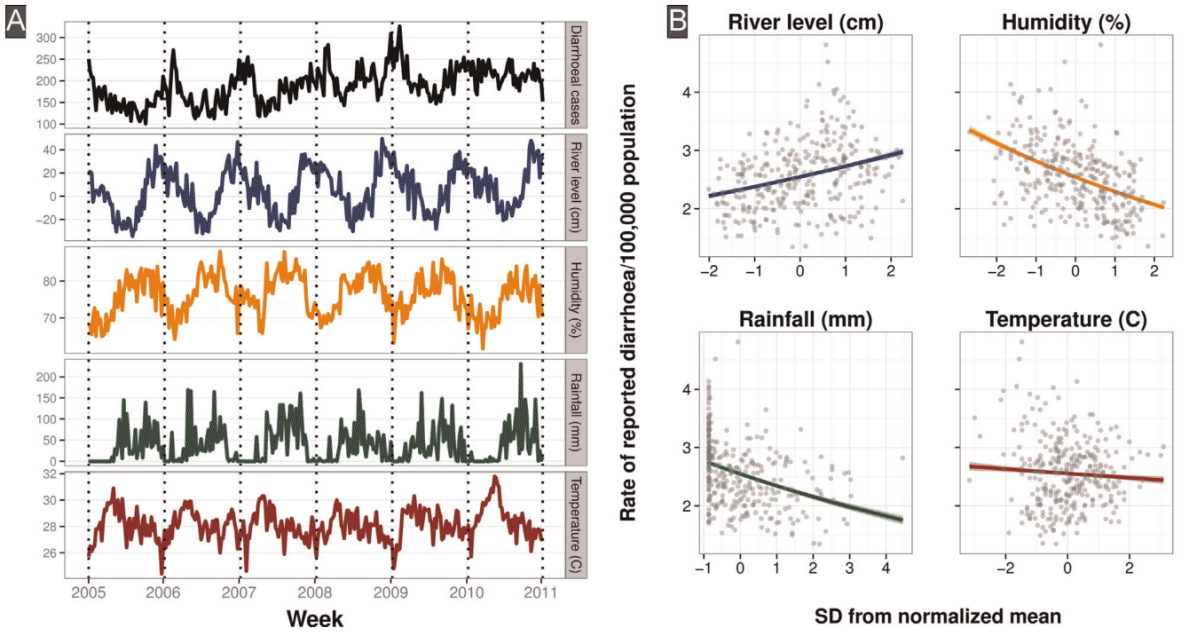

Fig. 1. Time series and correlation of diarrheal case counts and climate factors in Ho Chi Minh City. (A) From top to bottom: Individual weekly time series (period 2005–2010) of total citywide reported cases of diarrhea recorded at the three study sites, average river level of the Don Dien river in southern HCMC in cm, average weekly relative percent humidity, average weekly rainfall in cm and the average weekly temperature in Celsius. (B) Scatterplots of weekly diarrheal case counts and normalized average weekly river level and citywide humidity, rainfall and temperature. The climate variables have been normalized to zero mean and unit variance. The colored lines represent the fitted Poisson model. (For interpretation of the references to color in this figure legend,the reader is referred to the web version of this article.)

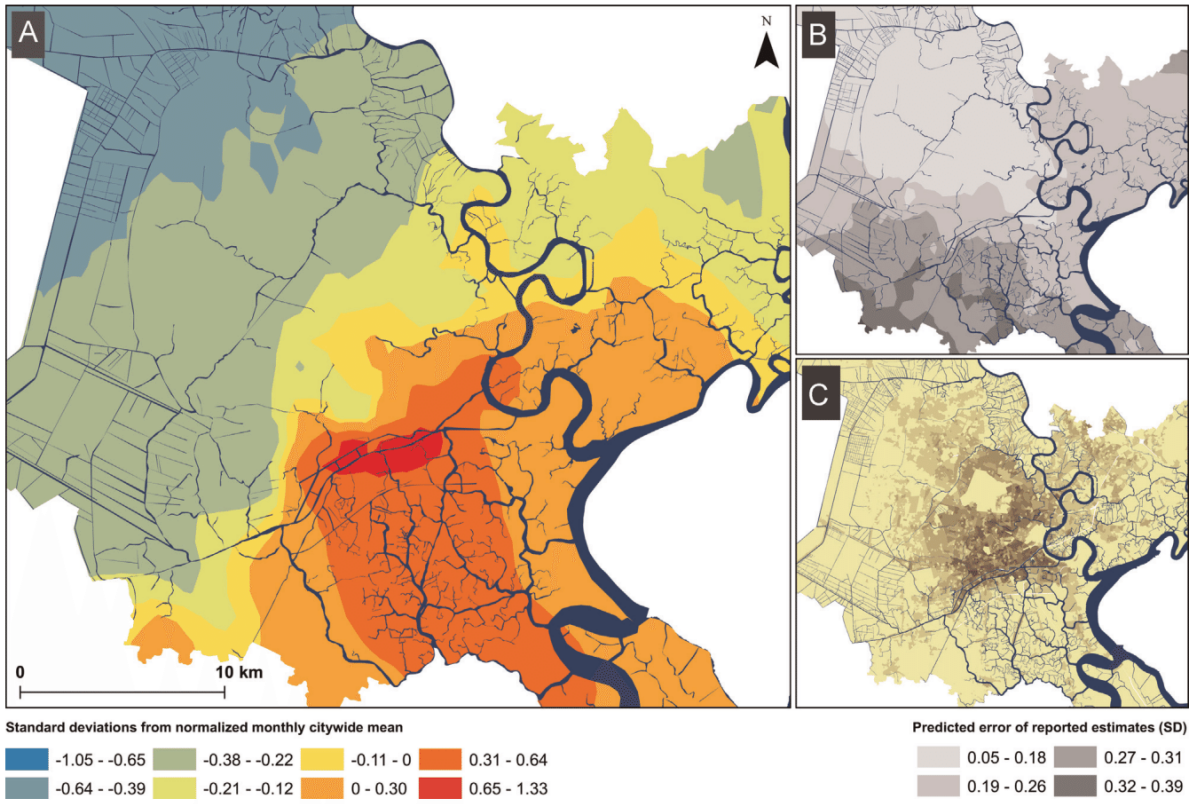

Fig. 2. The rate of reported diarrhea in Ho Chi Minh City, 2008–2010. (A) Map of HCMC showing the smoothed rate of reported diarrheal cases per 100,000 population by ward, with the scale in units of standard deviations from normalized monthly citywide mean. Districts are labeled by number in black. (B) Map showing the corresponding population density of HCMC, with darker colors indicating higher densities per square kilometer. (C) Map showing the predicted error of reported diarrheal rate estimates across HCMC, with darker colors indicating increasing uncertainty (scale shown in bottom right of figure, interpreted as standard deviations from predicted local estimate). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

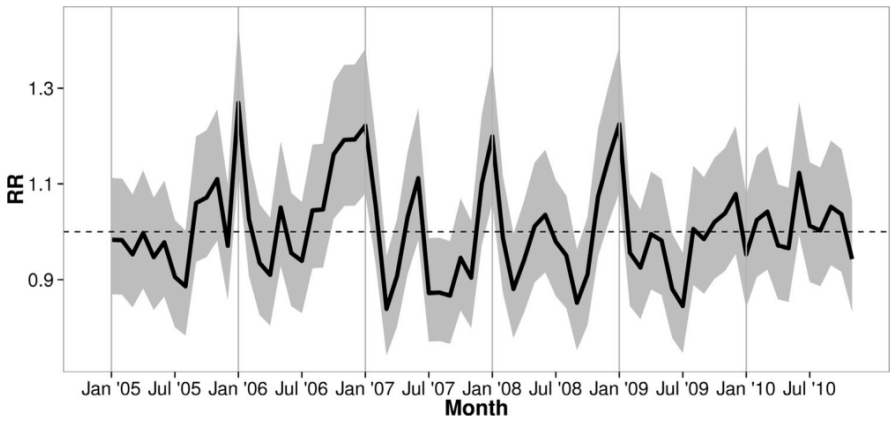

Fig. 3. The citywide monthly relative risk of diarrheal disease across Ho Chi Minh City. The solid line in the figure shows the relative risk (RR) of diarrheal disease, as compared to the population-level intercept in Table 1. The shaded region represents the 95% confidence interval for each of the monthly effects. The dashed line is a guide for assessing statistical significance; monthly effects spanning this line are significantly different from the average rate.

Confidence Interval (CI):1.06, 1.08), whereas humidity (RR:0.90, 95%CI:0.89, 0.91), rainfall (RR:0.92, 95%CI:0.91, 0.93) and temperature (RR:0.98, 95%CI:0.97, 0.99) were significantly negatively associated with the rate of diarrheal hospitalization throughout 2005–2010.

3.3. District-level model

In each year, the relative risk of reported diarrhea at the city level rose rapidly during the final weeks of the year, followed by a peak period lasting from 1 to 2 months. The citywide intercepts for each month of the study are highlighted in Fig. 3. Further, the elevation of each district was strongly related to the reported rate of diarrheal illness (Table 1 and Fig. 4). The RR of a one-meter increase in elevation was 0.95 (95%CI:0.92, 0.97). Therefore, the district with the lowest median elevation (0.32 m) experienced nearly double the risk of the highest district (7.95 m) (RR=0.57, 95%CI:0.31, 0.74). In further models incorporating both elevation and waterway coverage as fixed effects, the influence of waterways was non-significant, although this became significant when elevation was excluded. However, the inverse relationship between average district elevation and the proportion of district area covered by waterways (Pearson's $R=-0.59$, 95%CI: -0.80, -0.25) suggest that waterway coverage may explain some of the risk associated with lower elevation. Finally, district-level elevation was not associated with distance from CH1 ($\beta=0.07$, 95% CI$=-2.76$, 2.90), suggesting that adjustment for distance to the largest and most centrally located hospital does not confound the effect of elevation.

Table 1
Fixed effect coefficients from the mixed-effect model.

Variable	RR	95% CI	*p*
Intercept	1.9777	1.608, 2.432	< 0.0001
Elevation	0.9478	0.922, 0.974	0.00016
Log(CH1 Distance)	0.9687	0.902, 1.040	0.38026
Lag 1 Week	1.0464	1.039, 1.053	< 0.0001
Lag 2 Week	1.0258	1.019, 1.033	< 0.0001
Lag 3 Week	1.0134	1.006, 1.020	0.00014
Lag 4 Week	1.0145	1.008, 1.022	< 0.0001
Lag 5 Week	1.0071	1.000, 1.014	0.04378
Lag 6 Week	1.0197	1.013, 1.027	< 0.0001
Lag 7 Week	1.0095	1.003, 1.016	0.0074
Lag 8 Week	1.0057	0.999, 1.013	0.10213

RR: relative risk; CH1: Children's Hospital 1.

After accounting for seasonal and district-level effects, heterogeneity in the influence of weather and climate variables at the district level remained (Fig. 5 and Fig. S4). The district-level effect of 1SD increase in flooding on risk of diarrhea was significant for districts 6 (RR:1.02, 95%CI:1.01, 1.04), 8 (RR:1.04, 95%CI:1.02, 1.06) and 13 (RR:1.03, 95%CI:1.02, 1.05); located in the central/southwestern regions of HCMC (Fig. 5A). To understand how these effects impact risk, a change from the minimum observed river level (-2SD) to the maximum (2.3SD) in district 8, for example, correspond to a roughly 20% increase in incidence (RR=1.2, 95%CI:1.1, 1.3), whereas in district 15, the same change is not associated with a significant change in disease risk (RR=0.91, 95%CI:0.8, 1.0) (Table S3). We found that increasing humidity was inversely associated with reported diarrheal disease incidence in most districts of the city, particularly in the northeastern districts (Fig. 5B). District 8 was an exception to this trend, in which there was a positive association between diarrheal hospitalization and humidity (RR:1.03, 95%CI:1.02, 1.05). The citywide average and district-level effect of rainfall was non-significant across most districts (Fig. 5C). Finally, the direction of district-level association between temperature and risk varied across districts. In some districts (6, 8, 13, 14 and 23) this association was positive, whereas in others (districts 1, 4, 12, 13, 16, 18, 21 and 24) the effect appeared to be protective (Fig. 5D and Table S2). A change from the minimum observed temperature (-3.17SD) to maximum (3.09SD) in district 8, for example, would have an RR of 1.37 (95%CI:1.3, 1.5) whereas in district 4 this same change is associated with a slight protective effect (RR =0.84, 95%CI:0.8, 1.0).

4. Discussion

Our estimates of how climate and environmental factors impact diarrheal disease risk across HCMC can inform future climate-based early warning systems for diarrhea epidemics. We highlight substantial spatial heterogeneity in patterns of hospitalized diarrhea across districts of HCMC and show that this heterogeneity is driven in part by local variation in the impact of weather and environmental factors. Our multivariate analysis strongly indicates that the increased rates of hospitalization from low-lying areas is a consequence of local environmental conditions in these districts rather than their proximity to the hospitals that provided the data for these analyses. Future climate-based forecasting of diarrheal disease epidemics in other settings should incorporate the localized and heterogeneous nature of risk to allow for efficient

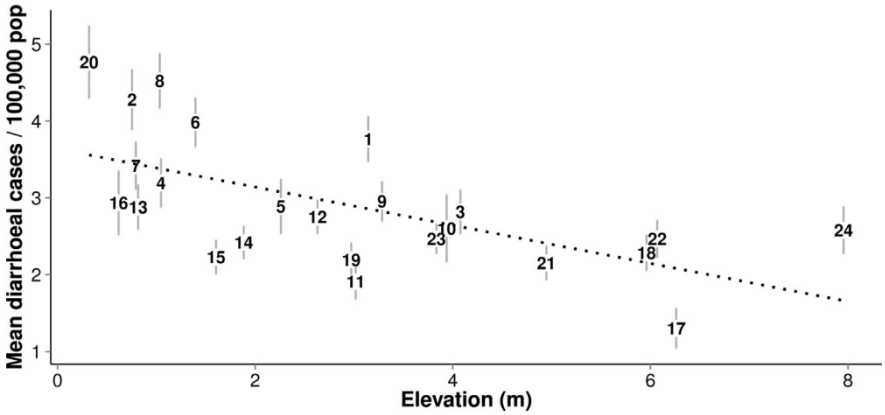

Fig. 4. The relationship between the predicted rate of reported diarrheal cases and elevation in Ho Chi Minh City, 2008–2010. Scatterplot showing the estimated average reporting rate by district for 2008–2010 (with bars indicating 95% credible interval) and average district elevation in meters above sea-level. The numbers within the plot represent the point estimate for each of the corresponding districts. The dashed line represents the fitted linear model.

targeting of scare resources such as preemptive rotavirus vaccination or provision of water filtration devices (Akanda et al., 2014; Lantagne and Clasen, 2012).

After adjusting for citywide monthly effects, we identified several spatial patterns in the impact of environmental factors on diarrheal disease risk. Southwestern HCMC was more likely to experience an increase in diarrheal hospitalizations during periods with increased river level, relative humidity and temperature. In the northeast of HCMC, however, diarrhea was reported more frequently during periods of low humidity. District 8 was consistently at high risk due to a number of factors including warmer air temperature, flooding, and humidity, making this region particularly vulnerable to predicted effects of climate change. Areas of the city most sensitive to variation in air temperature, such as district 8, may be more likely to experience bacterial or parasitic diarrheal disease, due to the enhanced growth and survival of bacterial and parasitic pathogens in warmer, wetter conditions (Rose et al., 2001).

District elevation was a consistent risk for all-cause diarrhea in this setting. HCMC is low lying, with 40–45% of land lying between 0 and 1 m above sea level (Asian Development Bank, 2010). Because low elevation and surface water coverage are so highly correlated in this setting, it is likely that the effect of low elevation on diarrhea risk is partially facilitated by increased exposure to contaminated surface and ground water (Wust et al., 2002). Flooding events are well documented to increase the risk of childhood diarrhea due to drinking water contamination (Ten Veldhuis et al., 2010; Wu et al., 2013). The canal water in HCMC is known to be heavily contaminated with human and animal waste and heavy metals due to limited restrictions on industrial and domestic waste discharge (Ha et al., 2008; Wust et al., 2002). The poorest residents, who often live in settlements along canals in low-lying districts near central HCMC, normally resort to use of such groundwater through drilled wells (Wust et al., 2002). The strong effect of low elevation, compared to those of local climate, suggests that persistent exposure to contaminated ground water plays a major role in diarrheal risk with effects that are amplified by seasonal variation in climate.

A study by the Asian Development Bank (ADB) highlighted the central districts of 4, 6 and 8 as most at risk of extreme flood events in the future and that this vulnerability may have serious implications for the risk of diarrheal disease and child health in these areas (Asian Development Bank, 2010). Our findings suggest that districts 13, 14 and 23 may be additionally vulnerable to mid- to long-term changes in climate and increasingly volatile weather trends with respect to diarrheal diseases in children. To stymie this growth, future work should include etiological diarrheal surveillance studies to disaggregate pathogen-specific trends to tailor prevention mechanisms more appropriately. Further studies could also use the presented modeling framework to examine the influence of climate and environment on other syndromes recorded routinely at hospital such as pediatric febrile and respiratory infections.

An important limitation of our findings is that our analysis relies on hospitalized diarrhea only. We are, therefore, unable to capture the factors that drive less severe, unreported cases that occur in the community. The trends we report are therefore applicable to our understanding of only the most severe diarrheal cases in the population who are able to attend the selected healthcare facilities. Furthermore, many of our cases had only district of residence recorded in the hospital report so we were unable to perform our analysis on a ward scale. Secondly, our results may be impacted by variation in socioeconomic status (SES) at the household and district level, which we were not able to observe. While measures of average household income exist for HCMC at the city level, reliable data on income and other measures of quality of life at higher resolution, i.e. at the district level, are not available. Recent work highlights how SES factors may impact susceptibility to environmental drivers of risk (Leckebusch and Abdussalam, 2015). In our data, households with low SES may be more likely to live in regions of HCMC with low elevation and poor WASH infrastructure; low SES may thereby drive the true risk of diarrheal disease. The frequency of health seeking behavior may also be correlated with SES and thus regions with lower SES, particularly those in semi-rural areas, may be underrepresented, although this issue is likely mitigated by adjustment for travel distance to central HCMC. However, the use of a random effects model allows us to capture spatial variation in risk that may be due to these unobserved socioeconomic factors. In addition, studies in other low-income, developing contexts with inadequate sanitation have shown that living at low elevation is often associated with low SES, as a consequence of the flooding and disease risk these areas are often subject to (Baker et al., 2011). Nonetheless, the absence of these data highlight the critical need for systematic collection of high-resolution socioeconomic data alongside the types of environmental and hospital and health information presented here.

In conclusion, we identified considerable spatial heterogeneity in patterns of all-cause diarrheal disease in HCMC as well as meaningful district-level variation in sensitivity to climate and

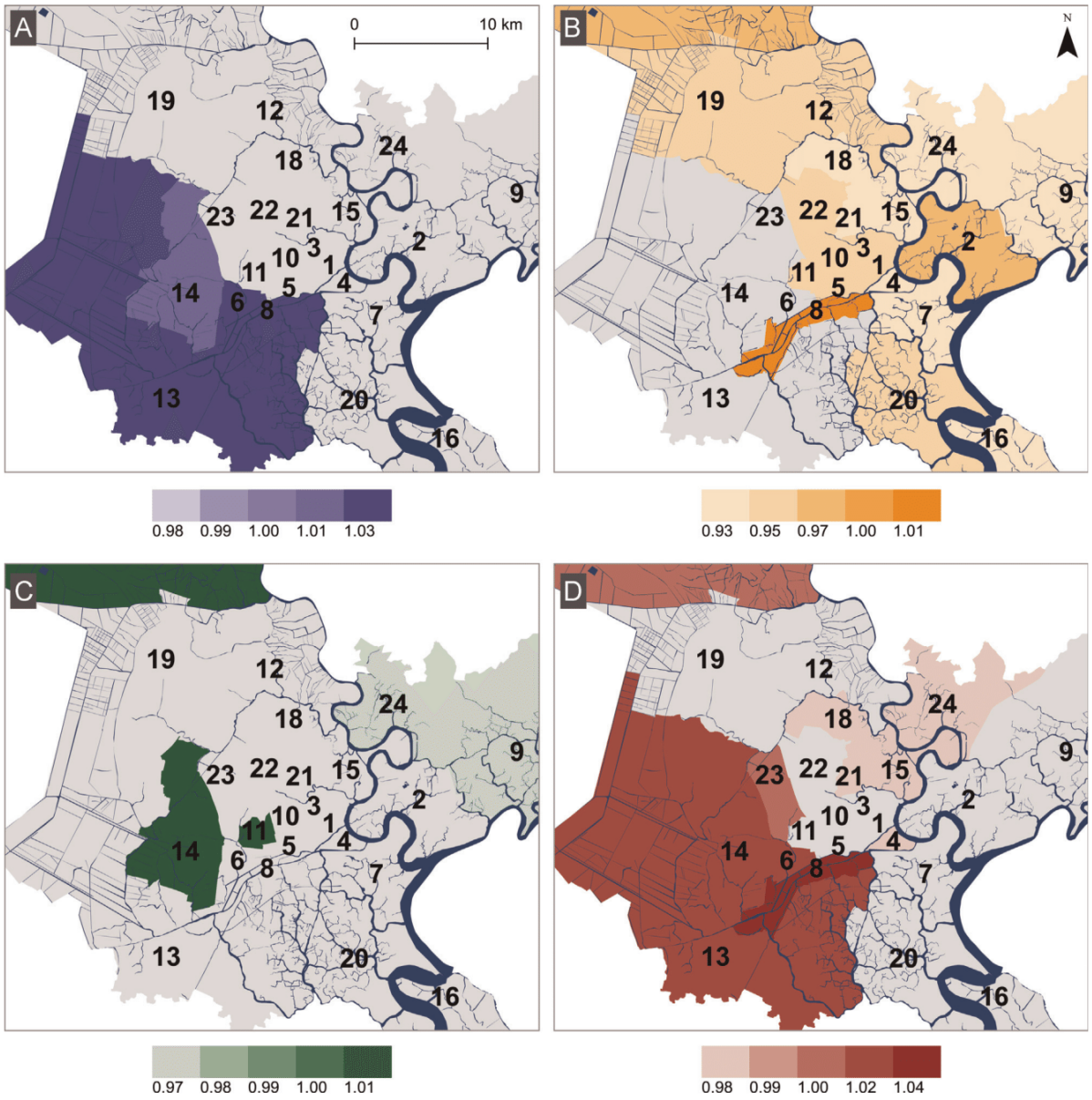

Fig. 5. District-level effects of weather and climate variables on diarrheal disease in Ho Chi Minh City. Maps showing the estimated effect scaled to standard deviation of each mean standardized climate variable (A) average weekly river level, (B) humidity, (C) rainfall and (D) temperature across the districts of HCMC. Intensity of color represents the magnitude of estimated coefficients, as indicated by legends. Gray areas indicate non-significant effects. Blue lines in the maps represent rivers and canals. Black numbers are district identifiers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

environmental factors. We focused on these citywide and localized patterns and drivers of this multi-pathogen syndrome with the aim of informing future climate-based predictive models of diarrheal disease in similar settings. Effective forecasting models of diarrhea in this setting would be a valuable tool when combined with existing or strengthened treatment protocols, breastfeeding promotion, and rotavirus immunization for effective and targeted reduction of diarrheal disease in children. Furthermore, quantification of the effects of weather and environment on risk of pediatric diarrheal disease will become increasingly important for future prevention and control policies, particularly in the face of predicted effects of climate change in both HCMC as well as other similarly high-risk settings (Asian Development Bank, 2010; Patz et al., 2005).

Conflicts of interest

The authors state that they have no conflicts of interest.

Ethical approval

Ethical approval for analysis of this anonymized data was granted by all local hospital ethical review committees (Children's Hospital 1, Children's Hospital 2 and the Hospital for Tropical Diseases) as well as the Oxford University Tropical Research Ethics Committee (OxTrec: 1045-13).

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Acknowledgment

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.healthplace.2015.08.001

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4.1 Supporting Information

Title: The impact of environmental and climatic variation on the spatiotemporal trends of hospitalized pediatric diarrhea in Ho Chi Minh City, Vietnam

Tables

Supplementary Table 1: District labels used in manuscript and district names of Ho Chi Minh City, Vietnam

District Label	District Name
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	Bình Chánh
14	Bình Tân
15	Bình Thạnh
16	Cần Giờ
17	Củ Chi
18	Gò Vấp
19	Hóc Môn
20	Nhà Bè
21	Phú Nhuận
22	Tân Bình
23	Tân Phú
24	Thủ Đức

Supplementary Table 2: District level effects of humidity (%), flooding (cm above or below mean), rainfall (mm) and temperature (^{0}C), relative risks (RR) and 95% confidence interval (CI) scaled to standard deviation of each mean-standardized climate variable.

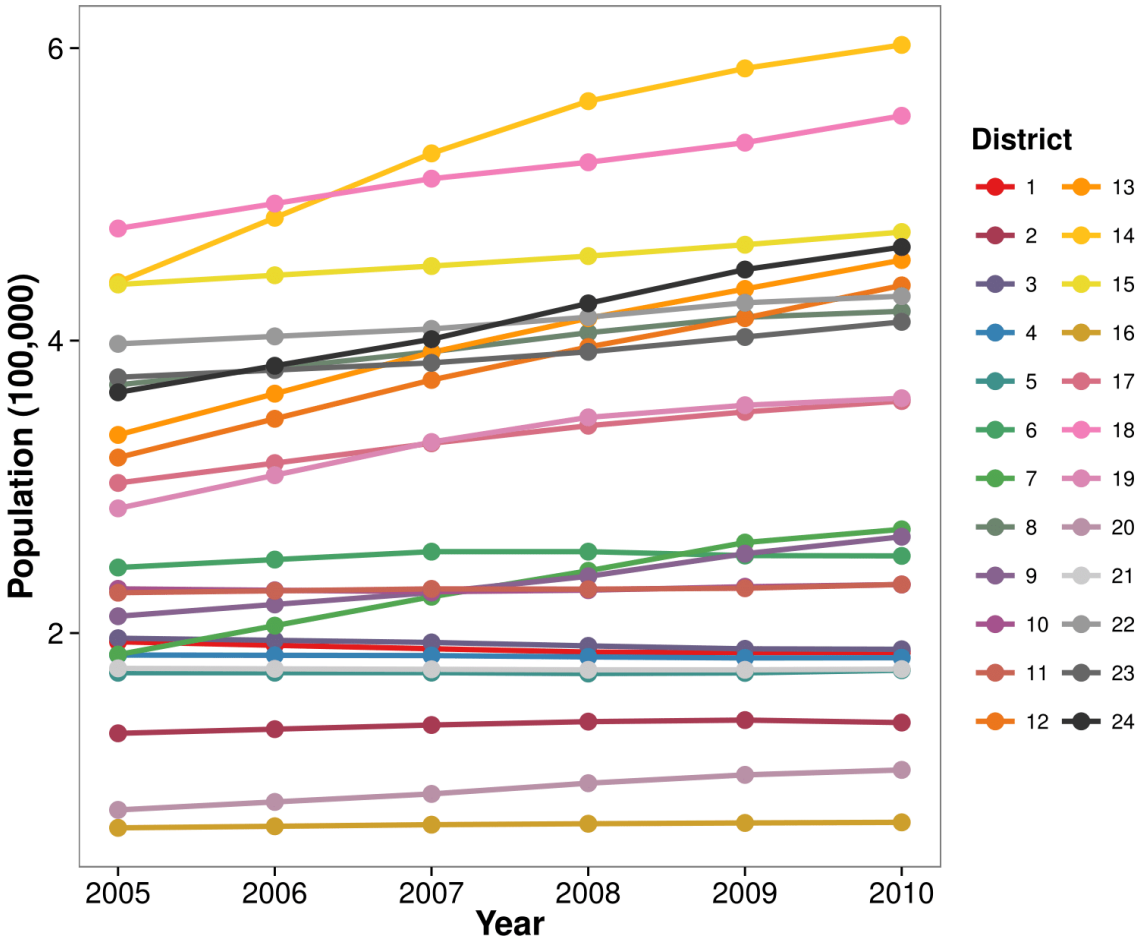
District	Humidity		Flooding		Rain		Temperature	
	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
1	0.972	0.955 , 0.989	0.985	0.967 , 1.002	1.007	0.985 , 1.029	0.992	0.981 , 1.004
2	0.975	0.957 , 0.993	0.991	0.973 , 1.009	1.019	0.997 , 1.043	0.999	0.987 , 1.011
3	0.960	0.941 , 0.979	0.992	0.974 , 1.011	1.012	0.988 , 1.036	0.993	0.981 , 1.006
4	0.938	0.921 , 0.956	0.990	0.972 , 1.008	0.998	0.976 , 1.021	0.982	0.970 , 0.995
5	0.974	0.954 , 0.994	1.004	0.984 , 1.023	1.001	0.977 , 1.026	1.008	0.994 , 1.021
6	1.012	0.993 , 1.030	1.024	1.006 , 1.043	0.989	0.967 , 1.012	1.035	1.022 , 1.048
7	0.952	0.935 , 0.968	1.006	0.988 , 1.024	0.997	0.976 , 1.019	0.997	0.985 , 1.009
8	1.032	1.016 , 1.049	1.038	1.020 , 1.056	0.982	0.962 , 1.001	1.051	1.039 , 1.063
9	0.946	0.929 , 0.964	1.003	0.985 , 1.022	0.976	0.955 , 0.999	0.991	0.979 , 1.003
10	0.966	0.947 , 0.986	1.006	0.987 , 1.025	0.996	0.972 , 1.020	1.005	0.992 , 1.019
11	0.956	0.936 , 0.976	0.998	0.979 , 1.018	1.026	1.000 , 1.051	1.001	0.987 , 1.014
12	0.960	0.945 , 0.976	0.989	0.972 , 1.006	1.006	0.986 , 1.027	0.992	0.981 , 1.003
13	0.993	0.976 , 1.010	1.034	1.015 , 1.052	0.993	0.972 , 1.014	1.034	1.022 , 1.047
14	1.004	0.988 , 1.021	1.018	1.001 , 1.036	1.021	1.000 , 1.042	1.033	1.021 , 1.045
15	0.935	0.920 , 0.949	0.983	0.967 , 1.000	1.015	0.995 , 1.036	0.980	0.969 , 0.991
16	0.950	0.927 , 0.974	0.988	0.968 , 1.008	1.005	0.977 , 1.033	0.987	0.972 , 1.002
17	0.978	0.957 , 0.999	1.003	0.984 , 1.023	1.027	1.001 , 1.053	1.015	1.001 , 1.029
18	0.941	0.927 , 0.955	0.987	0.970 , 1.004	0.989	0.970 , 1.008	0.980	0.969 , 0.991
19	0.960	0.942 , 0.978	1.000	0.982 , 1.019	1.000	0.978 , 1.023	1.000	0.988 , 1.013
20	0.970	0.950 , 0.991	1.006	0.987 , 1.025	0.985	0.961 , 1.010	1.003	0.990 , 1.017
21	0.941	0.921 , 0.961	0.989	0.970 , 1.008	1.005	0.980 , 1.030	0.984	0.971 , 0.997
22	0.964	0.949 , 0.980	0.998	0.981 , 1.016	1.006	0.986 , 1.027	0.998	0.986 , 1.009
23	0.987	0.969 , 1.004	1.012	0.994 , 1.030	1.012	0.990 , 1.034	1.019	1.007 , 1.031
24	0.942	0.927 , 0.957	1.004	0.987 , 1.022	0.967	0.947 , 0.986	0.986	0.976 , 0.998

Supplementary Table 3: District level relative risks (RR) and 95% confidence intervals (CI) for a change from the minimum level of each climate variable to the maximum level.

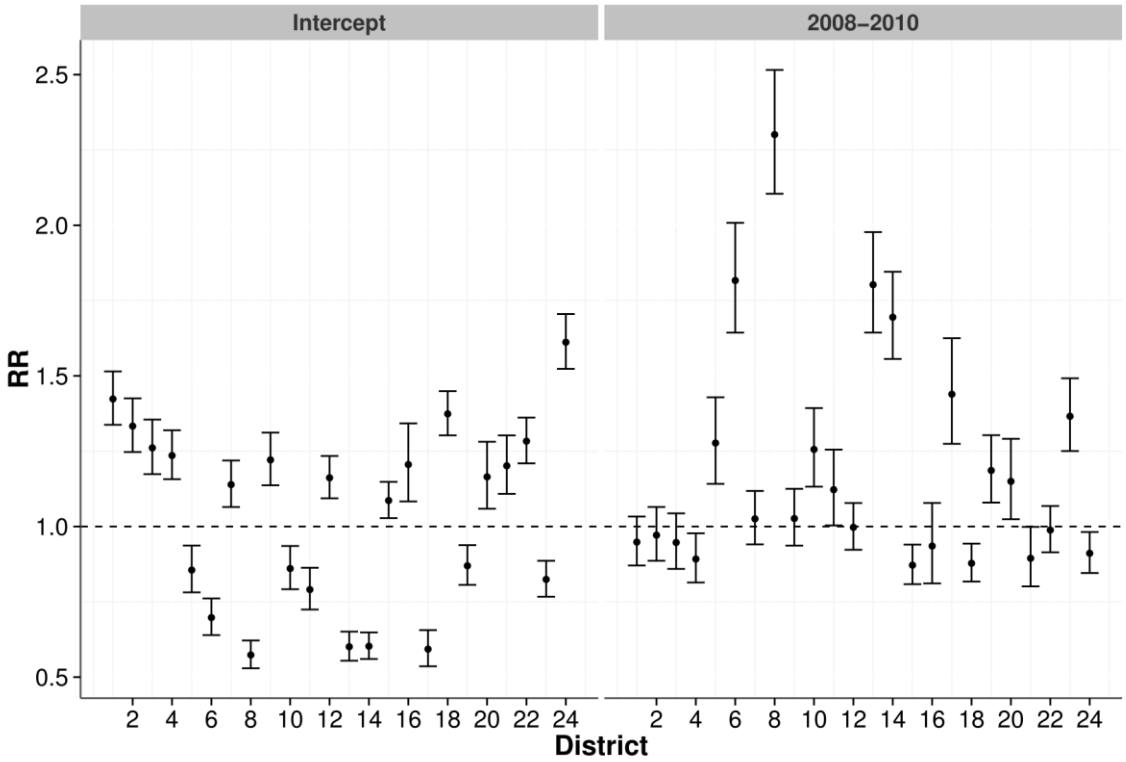
District	Humidity		Flooding		Rainfall		Temperature	
	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
1	0.833	0.732, 0.935	0.921	0.827, 1.010	1.044	0.905, 1.183	0.942	0.862, 1.029
2	0.851	0.744, 0.958	0.953	0.859, 1.047	1.120	0.981, 1.272	0.993	0.906, 1.080
3	0.762	0.649, 0.875	0.958	0.864, 1.058	1.076	0.924, 1.228	0.949	0.862, 1.044
4	0.631	0.530, 0.738	0.948	0.853, 1.042	0.987	0.848, 1.133	0.869	0.782, 0.964
5	0.845	0.726, 0.964	1.021	0.916, 1.120	1.006	0.855, 1.164	1.058	0.956, 1.152
6	1.071	0.958, 1.178	1.126	1.031, 1.225	0.930	0.791, 1.076	1.254	1.160, 1.349
7	0.715	0.613, 0.810	1.031	0.937, 1.126	0.981	0.848, 1.120	0.978	0.891, 1.065
8	1.190	1.095, 1.291	1.199	1.105, 1.293	0.886	0.760, 1.006	1.370	1.283, 1.457
9	0.679	0.578, 0.786	1.016	0.921, 1.115	0.848	0.716, 0.994	0.935	0.848, 1.022
10	0.798	0.685, 0.917	1.031	0.932, 1.131	0.975	0.823, 1.126	1.036	0.942, 1.138
11	0.738	0.619, 0.857	0.990	0.890, 1.094	1.164	1.000, 1.322	1.007	0.906, 1.102
12	0.762	0.673, 0.857	0.942	0.853, 1.031	1.038	0.912, 1.171	0.942	0.862, 1.022
13	0.958	0.857, 1.059	1.178	1.079, 1.272	0.956	0.823, 1.088	1.247	1.160, 1.341
14	1.024	0.929, 1.125	1.094	1.005, 1.189	1.133	1.000, 1.265	1.240	1.152, 1.327
15	0.613	0.524, 0.697	0.911	0.827, 1.000	1.095	0.968, 1.228	0.855	0.775, 0.935
16	0.703	0.566, 0.845	0.937	0.832, 1.042	1.032	0.855, 1.209	0.906	0.797, 1.015
17	0.869	0.744, 0.994	1.016	0.916, 1.120	1.171	1.006, 1.335	1.109	1.007, 1.211
18	0.649	0.566, 0.732	0.932	0.843, 1.021	0.930	0.810, 1.051	0.855	0.775, 0.935
19	0.762	0.655, 0.869	1.000	0.906, 1.100	1.000	0.861, 1.145	1.000	0.913, 1.094
20	0.822	0.703, 0.946	1.031	0.932, 1.131	0.905	0.754, 1.063	1.022	0.927, 1.123
21	0.649	0.530, 0.768	0.942	0.843, 1.042	1.032	0.874, 1.190	0.884	0.789, 0.978
22	0.786	0.697, 0.881	0.990	0.900, 1.084	1.038	0.912, 1.171	0.985	0.898, 1.065
23	0.923	0.816, 1.024	1.063	0.969, 1.157	1.076	0.937, 1.215	1.138	1.051, 1.225
24	0.655	0.566, 0.744	1.021	0.932, 1.115	0.791	0.665, 0.912	0.898	0.826, 0.985

Minimum and maximum mean-normalized values (1) humidity: -2.7SD, 2.2SD (2) flooding: -2.0, 2.3 (3) rainfall: -0.9, 4.5 (4) temperature: -3.2, 3.1

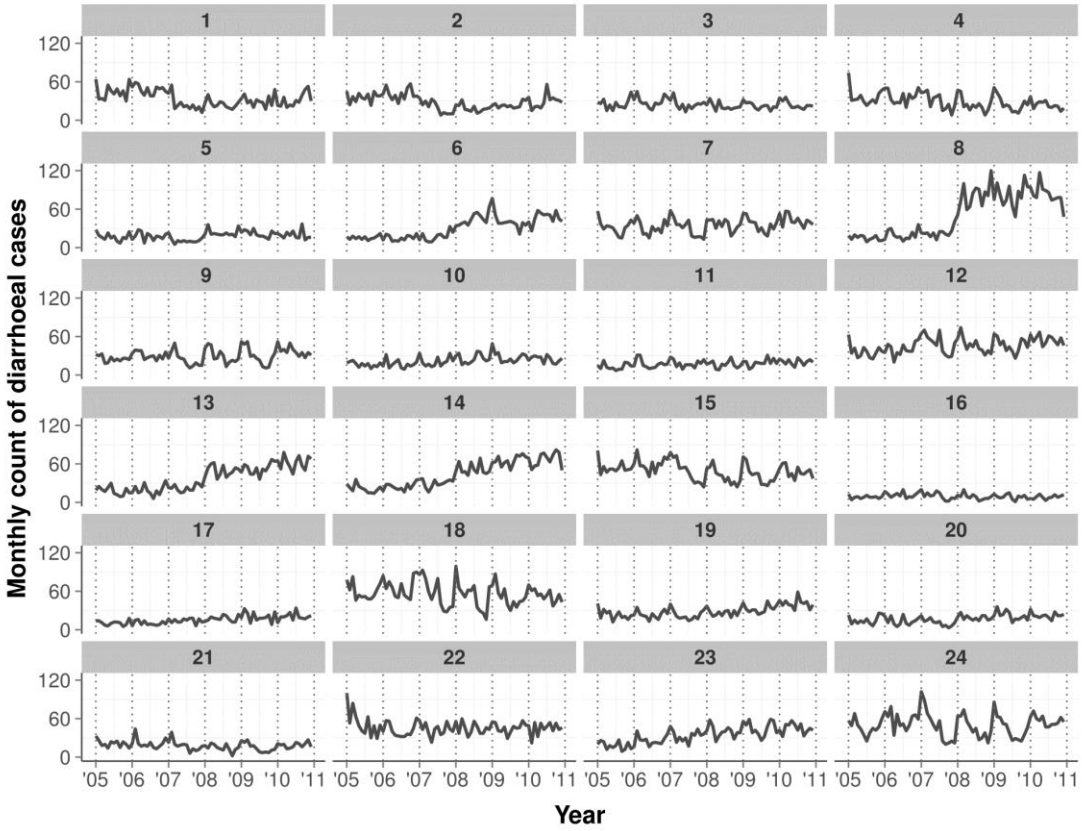
4.2 Supplementary Figures



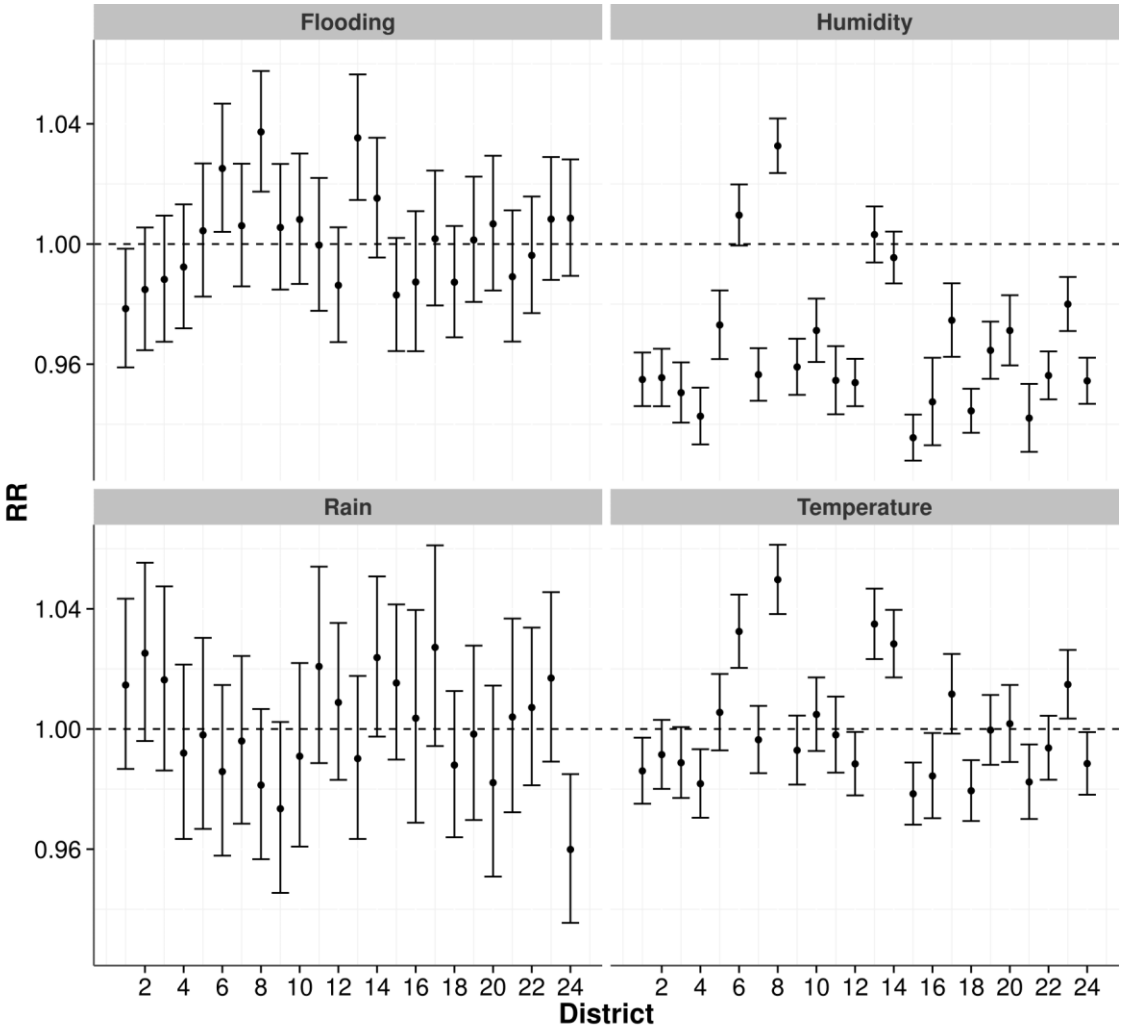
Supplementary Figure 1: **Population growth by district over time**. To account for changing population size over the period included in the analysis (2005-2010), we estimated weekly district-level population sizes using linear interpolation, assuming a constant rate of change in district population size between available census observations from 2005, 2008, 2009 and 2010 (*Statistical Yearbook of Ho Chi Minh City 2011*, 2012).



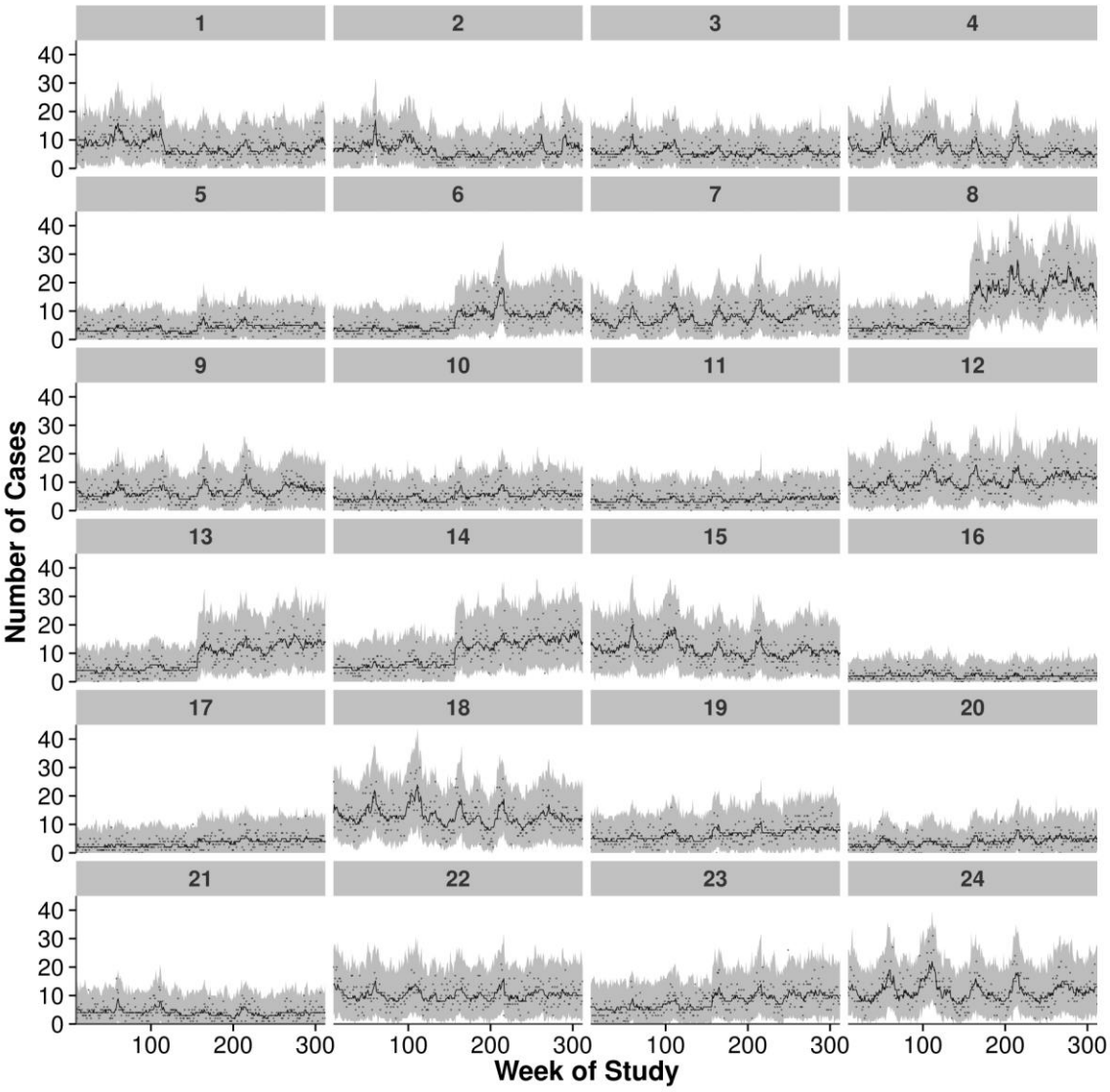
Supplementary Figure 2: District level random intercept and change in reporting from 2008-2010. (A) District level variability in baseline risk assumed to apply consistently over time, shown in relative risk in terms of deviation from overall average rate. (B) District level relative risk of reporting in any period after 2008, again shown in terms of deviation from overall average rate. Dashed lines in each panel are provided as a guide for assessing statistical significance.



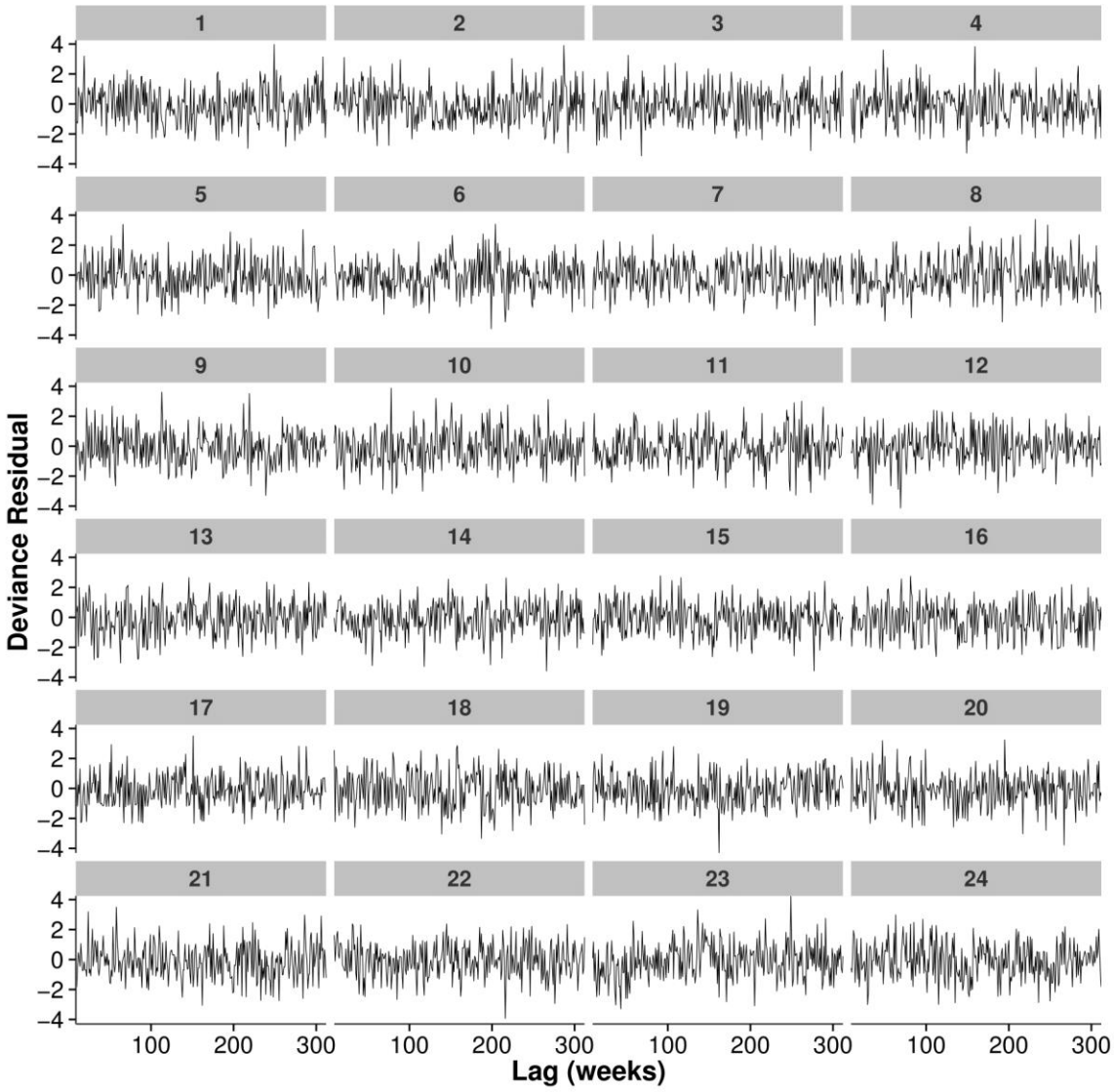
Supplementary Figure 3: District level time series. Count of diarrheal cases per week over the study period (2005-2010) by district.



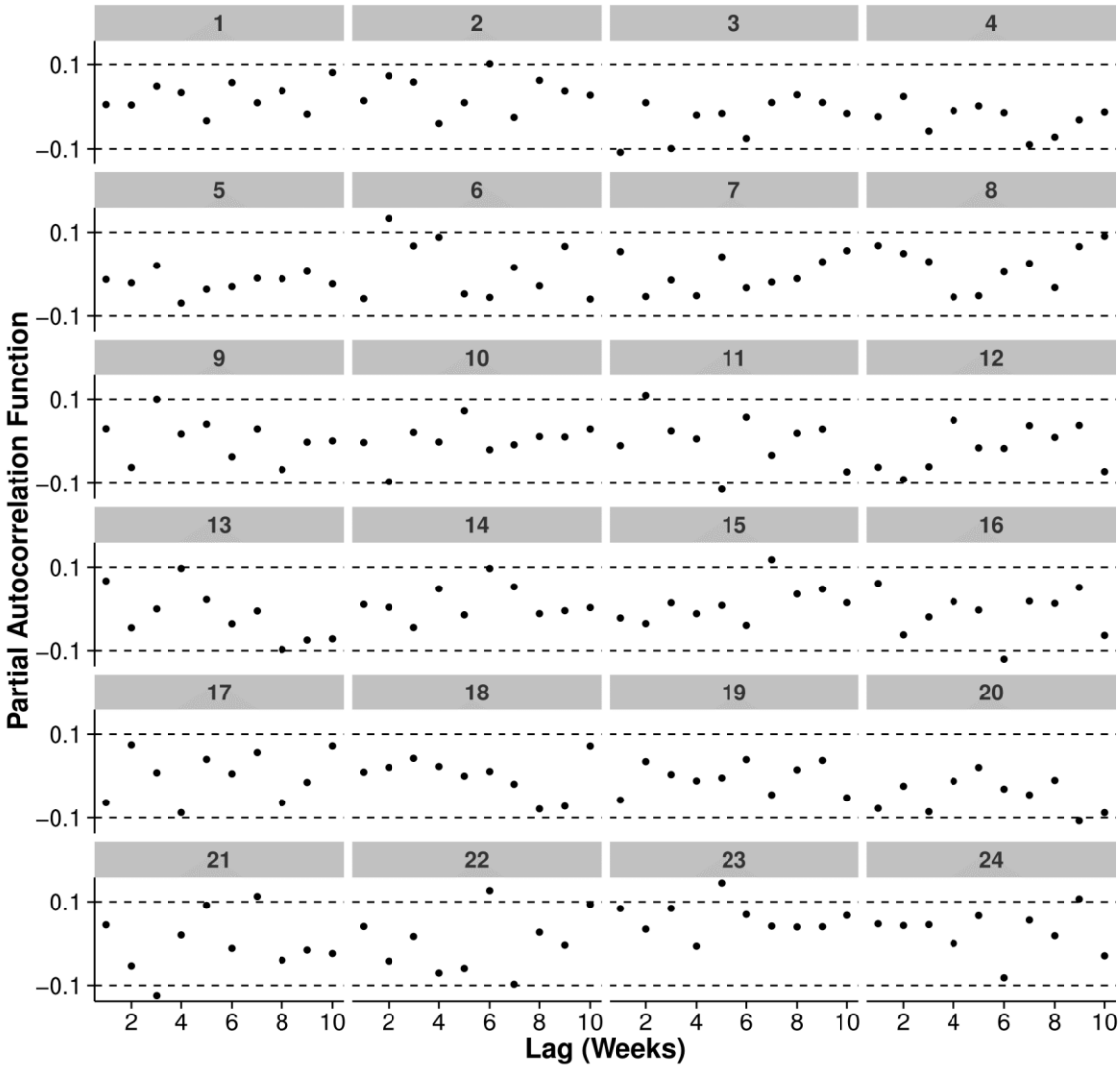
Supplementary Figure 4: District level weather and climate effects. Relative risks of flooding, humidity, rainfall and temperature by district predicted from the mixed effects model. The dashed line in each panel is provided as a guide for assessing statistical significance.



Supplementary Figure 5: **Goodness of fit by district.** Each panel illustrates the fit of the model to the data from each district using 1000 simulations from the fitted model. Points indicate the number of cases for each week. The solid line shows the median simulated value for each week, and the gray shaded area shows the range from the minimum to maximum simulated values.



Supplementary Figure 6: District level deviance residuals. Each panel of the plot shows the deviance residuals for each district after accounting for fixed effects, and seasonal and district-level random effects.



Supplementary Figure 7: Partial autocorrelation function. Partial autocorrelation function (PACF) of district-level residuals. Points in each panel of the plot show the PACF for each district at each week of a 10-week lag. Dashed lines at 0.1 and -0.1 show a range of values indicating minimal residual temporal autocorrelation.

5 RESEARCH PAPER 3: The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study

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RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	International Journal of Infectious Disease		
When was the work published?	2015		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	NA		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am co-first author, along with Dr Katherine Anders. Dr Anders set up the original birth cohort study in 2009 and oversaw the management, data and sample collection and data management of the entire cohort. I cleaned and collated all demographic and descriptive data relevant to the diarrhoea study from the large database. We both were

	responsible for cleaning and linking diarrhoeal episode data to illness visit records. I developed the STATA code and performed the regression analyses. I also performed the geospatial cluster analysis. I made all tables in the manuscript and Dr Anders generated Figures 1 and 2 for the manuscript. I drafted the manuscript and was responsible for the submission process.

Student Signature: _____ **Date:** 16 NOV 2015

Supervisor Signature: _____ **Date:** 16/11/15

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The epidemiology and aetiology of diarrhoeal disease in infancy in southern Vietnam: a birth cohort study

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SUMMARY

Objectives: Previous studies indicate a high burden of diarrhoeal disease in Vietnamese children, however longitudinal community-based data on burden and aetiology are limited. The findings from a large, prospective cohort study of diarrhoeal disease in infants in southern Vietnam are presented herein.
Methods: Infants were enrolled at birth in urban Ho Chi Minh City and a semi-rural district in southern Vietnam, and followed for 12 months (n = 6706). Diarrhoeal illness episodes were identified through clinic-based passive surveillance, hospital admissions, and self-reports.
Results: The minimum incidence of diarrhoeal illness in the first year of life was 271/1000 infant-years of observation for the whole cohort. Rotavirus was the most commonly detected pathogen (50% of positive samples), followed by norovirus (24%), Campylobacter (20%), Salmonella (18%), and Shigella (16%). Repeat infections were identified in 9% of infants infected with rotavirus, norovirus, Shigella, or Campylobacter, and 13% of those with Salmonella infections.
Conclusions: The minimum incidence of diarrhoeal disease in infants in both urban and semi-rural settings in southern Vietnam was quantified prospectively. A large proportion of laboratory-diagnosed disease was caused by rotavirus and norovirus. These data highlight the unmet need for a rotavirus vaccine in Vietnam and provide evidence of the previously unrecognized burden of norovirus in infants.

1. Introduction

Diarrhoea remains a substantial cause of morbidity and mortality amongst children globally.[1,2] In a study in rural central Vietnam, the incidence of diarrhoea in children under 5 years of age was found to exceed 115 episodes/1000 child-years.[3] Risk factors for diarrhoea in Vietnam include, as in many settings, male gender, age less than 2 years, and poor socioeconomic indicators such as household crowding and poor hygiene habits.[4,5] There are no equivalent population-based estimates of diarrhoea in southern tropical Vietnam, where approximately 40% of the country's population live.

A recent study in southern Vietnam illustrated the relative contributions of rotavirus, norovirus, and the bacterial pathogens

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Shigella spp, *Salmonella spp*, and *Campylobacter spp* as the aetiological agents of diarrhoea in hospitalized children under 5 years of age in Ho Chi Minh City (HCMC).[6] How well these data represent the community level burden of diarrhoeal disease is unclear. Further, these data suggest that the majority of hospitalized diarrhoea cases are in children <12 months of age,[6] which is the pivotal age group at which rotavirus vaccine should be targeted. Longitudinal community cohort studies provide an opportunity to evaluate the epidemiology and disease burden of diarrhoea to a fuller scale than hospital-based research. However, few studies have evaluated the incidence of diarrhoea in Vietnam,[3,7] and to date none have focused exclusively on the tropical south of the country. To address this knowledge gap, we sought to define the burden, aetiology, and risk factors for diarrhoeal disease through community cohorts of infants in two distinct settings in this densely populated, rapidly industrializing region. A better understanding of the epidemiology and aetiologies of diarrhoeal disease in southern Vietnam will inform rational public health interventions.

2. Methods

2.1. Description of the cohort

The cohort structure and methodology have been described previously.[8] Briefly, pregnant women were enrolled from 2009 to 2013 in southern Vietnam in two locations: women resident in central HCMC, the largest city in southern Vietnam, were enrolled at Hung Vuong Obstetric Hospital in HCMC; women resident in Cao Lanh District, Dong Thap Province, which is 120 km southwest of HCMC and situated in a semi-rural setting, were enrolled at Dong Thap Provincial Hospital. After delivery, infants were enrolled and followed up for the first 12 months of life with routine visits at 2, 4, 6, 9, and 12 months of age. A brief questionnaire detailing growth and illness in the preceding period since the last visit was administered, and a series of samples (blood, throat swab, nasopharyngeal swab) was collected at each routine visit.

2.2. Diarrhoeal episode detection

During the 12 months of follow-up, passive detection of diarrhoeal illness was performed, in which families were asked to take their child to a designated study clinic if the infant was unwell. At presentation, a brief clinical report was collected, as well as a stool sample. If the child was admitted, a detailed clinical evaluation was recorded. Blood samples were collected at the discretion of the treating physician. A new episode of diarrhoea was defined by ≥7 days between the onset dates of symptoms. Diarrhoea was defined as three watery loose stools or at least one bloody/mucoid diarrhoeal stool within 24 h,[9] or an increase in stool frequency as determined by the parent's judgement.

A secondary source of data on diarrhoeal episodes were self-reports by the mother of diarrhoeal illness in their infant for the period prior to each study visit.

2.3. Laboratory analysis

Stool samples collected from diarrhoeal episodes were stored at 4 °C until transport within 24 h and were then stored at −80 °C until further testing. One-step reverse transcriptase (RT) PCRs for rotavirus and norovirus genogroups I and II (GI and GII) were performed using RNA Master Hydrolysis Probes (Roche Applied Sciences, UK) on a LightCycler 480 (Roche Applied Sciences, UK) with the primers and probe sequences and PCR cycling conditions described previously.[10] Real-time PCR cycling conditions for Shigella (target *ipa*H) and Campylobacter (*Campylobacter jejuni* target: *hip*O; *Campylobacter coli* target: *gly*A) were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s, as described previously.[11,12] Salmonella was detected using an in-house assay targeting the *inv*A gene, which is conserved across the eight *Salmonella* subspecies, with cycling conditions as follows: 95 °C for 15 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 60 s. The sequences of the primers and probe for the *inv*A gene were as follows: forward 5′-TCATCGCACCGTCAAARGA-3′, reverse 5′-CGATTTGAARGCCGGTATTATT-3′, probe: 5′-FAM-ACGCTTCGCCGTTCRCGYGC-BHQ1-3′. The limit of detection was 5 copies/reaction. Stool samples were not available from self-reported diarrhoea episodes.

2.4. Statistical analyses

Two separate incidence measurements were calculated: one evaluating diarrhoeal presentations at a study clinic and/or admitted to hospital, and the other based solely on self-reported diarrhoeal illness derived from information collected at the routine follow-up visits. These data were not merged. Infant-years of observation (IYO) for each infant were derived from the date of birth and date of exit from the study due to either completion of follow-up, documented early withdrawal, or loss to follow-up, defined by the last routine visit or illness presentation, whichever was later, if the full 12-month follow-up period was not completed. Pathogen-specific incidence estimates were not calculated due to low counts, but the incidence of aetiological groups (bacterial, viral, or mixed infection) was evaluated. Comparisons between groups were made using the Kruskal–Wallis test for continuous variables with non-normal distributions and the Chi-square test for categorical variables.

Multivariable negative binomial regression was used to identify risk factors associated with severe diarrhoea presenting to a study clinic and/or admitted to hospital. Regression was performed independently for each study site due to the heterogeneity in risk profiles between HCMC and Dong Thap. Factors were included in the multivariable model according to hypothesized associations determined a priori (maternal characteristics, socioeconomic indicators, household elevation), as well as those found to be significantly associated in the univariable analysis ($p < 0.05$). All analyses were performed in Stata v. 13 (StataCorp, College Station, TX, USA).

2.5. Spatial clustering analyses

To investigate the presence of spatial clustering of diarrhoeal illness, we used a Bernoulli model with all diagnosed episodes of diarrhoea as cases, and children without any reported history of diarrhoeal episodes as the background population using SaTScn v. 9.1.1 (http://www.satscan.org/). Each pathogen in turn was also considered as a case, with the control group remaining all children in the cohort with no reported episode. For the analyses, the upper limit for cluster detection was specified as 50% of the study population. The significance of the detected clusters was assessed by a likelihood ratio test, with a *p*-value obtained by 999 Monte Carlo simulations generated under the null hypothesis of a random spatiotemporal distribution.

2.6. Ethics

Four hospitals in HCMC (Hospital for Tropical Diseases, Hung Vuong Obstetric Hospital, District 8 Hospital, Children's Hospital 1) and Dong Thap Provincial Hospital participated in the study. The protocol was approved by the institutional review boards of all these hospitals, as well as the Oxford Tropical Research Ethics

Committee. Written informed consent was obtained from all participants.

3. Results

3.1. Baseline characteristics of the cohorts

From July 2009 to December 2013, a total of 6706 infants were enrolled in the birth cohort from 6679 mothers (27 sets of twins). A total of 6239.4 infant-years of observation (IYO) were recorded for these children. In Dong Thap, there were 2458 infants enrolled with 2199.4 IYO, and in HCMC there were 4248 infants enrolled with 4040 IYO. The full 12-month follow-up was completed by 87% of the cohort, with 33% (289/884) of early exits occurring after at least 9 months of cohort membership. Slightly over half of enrolled babies were male (52%), with roughly 5% being of low birth weight (<2500 g) (Table 1). The majority of children (91%) were breastfed after birth; 33% were exclusively breastfed. The use of milk formula after birth was more frequently reported in HCMC (92%) compared with Dong Thap (26%). Households in Dong Thap were more likely to have characteristics of lower socioeconomic status compared to HCMC, with a higher prevalence of household crowding, a lack of flush toilets, use of river water as the primary water source, and lower maternal education level (Table 1).

Table 1
Baseline characteristics of Ho Chi Minh City (HCMC) and Dong Thap participants

Characteristic	HCMC	Dong Thap	Total
Total number of infants	4248	2458	6706
Infant years of follow-up	4040	2199	6239
Maternal			
Age at delivery, years	28 (24–32)	25 (22–29)	27 (23–31)
Education			
Lower secondary or below	2555 (60.2)	1894 (77.1)	4449 (66.4)
Higher secondary or above	1692 (39.8)	561 (22.9)	2253 (33.6)
Infant			
Male sex	2248 (52.9)	1255 (51.1)	3503 (52.2)
Low birth weight[a]	199 (4.7)	110 (4.5)	309 (4.6)
Rotavirus vaccine[b]	1035 (24.4)	0 (0)	1035 (15.4)
Breastfed after birth?			
Yes, exclusively	364 (8.6)	1823 (74.2)	2187 (32.6)
Yes, plus formula	3299 (77.7)	620 (25.2)	3919 (58.4)
No, only formula	585 (13.8)	15 (0.6)	600 (8.9)
Started food before 6 months	1715 (42.2)	238 (11.2)	1953 (31.6)
Older sibling	2476 (58.3)	1161 (47.3)	3637 (54.3)
Household			
Elevation ≤3 m	996 (23.4)	207 (8.4)	1203 (17.9)
Household crowding[c]	2727 (66.8)	1701 (77.4)	4428 (70.5)
Toilet type			
Own flush	3704 (87.2)	1394 (56.8)	5098 (76.1)
Shared flush	524 (12.3)	63 (2.6)	587 (8.8)
None/bush	1 (0)	599 (24.4)	600 (9.0)
Other	18 (0.4)	398 (16.2)	416 (6.2)
Water source			
Piped to home	2844 (67.0)	761 (31.0)	3605 (53.8)
Piped to public tap	51 (1.2)	277 (11.3)	328 (4.9)
Bottled	1292 (30.4)	190 (7.7)	1482 (22.1)
River/stream	1 (0)	1145 (46.7)	1146 (17.1)
Other	59 (1.4)	81 (3.3)	140 (2.1)
Water treatment			
Boil	2642 (62.2)	1662 (67.7)	4304 (64.2)
Filter	282 (6.6)	582 (23.7)	864 (12.9)
None	1311 (30.9)	193 (7.9)	1504 (22.4)
Other	12 (0.3)	17 (0.7)	29 (0.4)
Pigs in household	16 (0.4)	242 (9.9)	258 (3.8)
Poultry in household	134 (3.2)	761 (31.0)	895 (13.4)

[a] <2500 g.
[b] One or more doses.
[c] Two or more people per room.

3.2. Incidence of diarrhoeal disease

During the follow-up period there were 1690 diarrhoeal presentations detected through clinic-based surveillance. The majority of these illnesses were treated on an outpatient basis (91.4%). The minimum incidence of diarrhoeal presentations estimated for the cohort as a whole was 271/1000 IYO. In Dong Thap, this figure was 604.3/1000 IYO and in HCMC was 89.4/1000 IYO. The minimum incidence estimates for hospitalized diarrhoeal illness in each location were 57.3/1000 IYO and 4.5/1000 IYO, respectively. There were 1656 self-reported diarrhoeal episodes at routine follow-up visits, corresponding to an incidence of 265.4/1000 IYO for the entire cohort. The incidence of self-reported diarrhoea was similar between the study sites: in Dong Thap it was 318.3/1000 IYO and in HCMC it was 236.6/1000 IYO.

3.3. The aetiology of diarrhoeal disease

Of the 1690 unique diarrhoeal presentations, 1309 (77%) had a corresponding stool sample. Of these, a total of 748 (57%) tested positive for one or more of the six pathogens screened. Among the positive samples, rotavirus was the most commonly detected pathogen (53%, 395/748); notably almost a third of these samples were also positive for another pathogen (32%, 128/395). Norovirus was identified in 24% (176/748) of positive samples, of which 88 (50%) samples were also positive for another pathogen. PCR amplifications consistent with the presence of Shigella, Salmonella, and Campylobacter were identified in 16% ($n = 117$), 18% ($n = 135$), and 20% ($n = 152$) of samples, respectively. Amongst the norovirus infections, GII was predominant (167/176, 95%), with an additional two samples positive for both GI and GII. Of the Campylobacter infections, *C. jejuni* was detected most often (94%, 143/152), followed by *C. coli* (6%). Mixed infections accounted for 26% (192/748) of all positive samples, most of which (68%, 130/192) were a mixed viral/bacterial infection.

Stool samples were collected from a far greater proportion of diarrhoeal episodes in the Dong Thap cohort compared to the HCMC cohort (86% vs. 47%). For inpatient diarrhoeal episodes in particular, the completeness of stool sample collection was far higher in Dong Thap (103/126; 82%) than in HCMC (5/18; 28%). This was due to difficulties in identifying hospital admissions of cohort members in real time in HCMC. The proportion of samples positive for at least one pathogen did not differ between sites, but was collectively higher among inpatient samples than outpatient samples (69% vs. 56%). The distribution of aetiologies differed significantly between HCMC and Dong Thap (Chi-square $p < 0.001$), with viral infections more common in HCMC and bacterial and mixed viral/bacterial infections more common in Dong Thap (Figure 1A). Mixed viral/bacterial infections were more common among hospitalized diarrhoeal cases than outpatients, however the overall distribution of aetiologies was not significantly different between outpatients and inpatients (Chi-square $p = 0.09$; Figure 1B). Among all detected diarrhoeal episodes, infections with a mixed viral/bacterial aetiology were most likely to be admitted to hospital (26%, 35/133), followed by viral infections (17%, 67/391).

Repeat infections with the same pathogen were identified in a subset of infants. Rotavirus was identified in 365 infants, 32 of whom (9%) had at least two discrete rotavirus infections separated by at least 7 days. This proportion was the same for norovirus (15/163), Shigella (10/108), and Campylobacter (12/141). Of the 120 infants with Salmonella infection, 15 (13%) had at least two distinct episodes where Salmonella was detected. Figure 2 shows the distribution of the interval between repeated infections, by pathogen. The median interval between repeated infections ranged from 37 days for Salmonella to 106 days for norovirus,

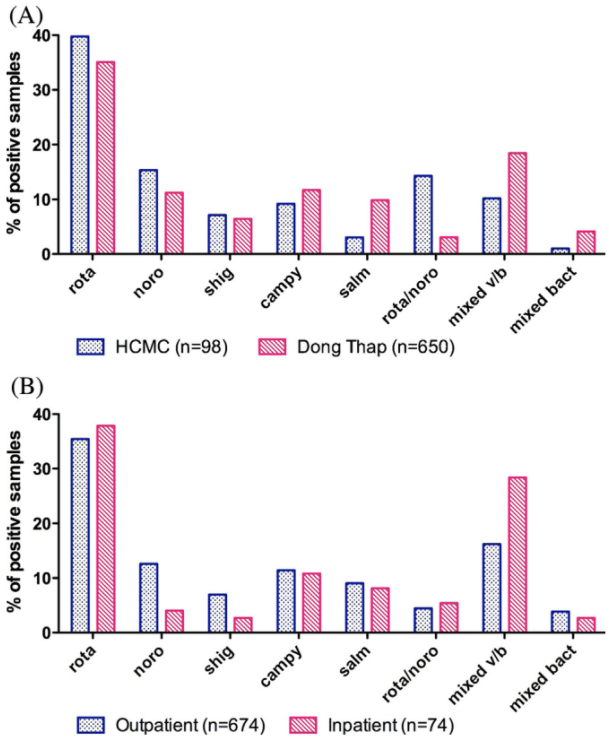

Figure 1. Aetiology of diarrhoeal disease. The proportion of diarrhoeal episodes (among those with a positive stool sample) that were positive for each single pathogen or combination of pathogens, (A) by study site, and (B) by hospitalization status.

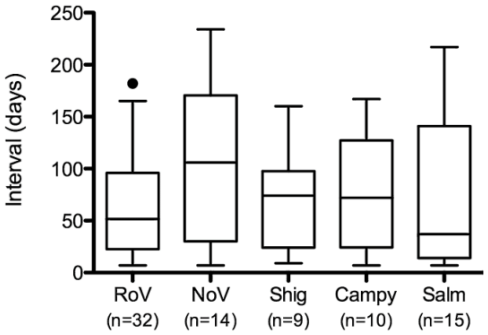

Figure 2. Time interval between repeated infections with enteric pathogens. Each box-and-whiskers plot shows the distribution of time intervals (in days) between repeated infections with the same enteric pathogen. Boxes indicate the median and interquartile range, and the whiskers indicate the 5th and 95th percentiles. RoV, rotavirus; NoV, norovirus; Shig, Shigella; Campy, Campylobacter; Salm, Salmonella. The numbers below each pathogen label indicate the total number of secondary or tertiary infections for that pathogen.

but this difference was not statistically significant (Kruskal–Wallis $p = 0.44$).

3.4. Clinical characteristics by aetiological group

Amongst all 1690 diarrhoeal episodes detected by clinic-based surveillance, the median age of the affected infants was 6.5 months (interquartile range (IQR) 4.6–8.7 months). A total of 55% ($n = 934$) of all diarrhoeal cases were male. Amongst episodes with an identified aetiology, infants with mixed infections tended to be slightly older (median 8 months) compared to those with the other aetiological groups (Table 2). The median axillary temperature at hospital admission was 37.8 °C (IQR 37–38.5 °C), which did not

Table 2
Incidence and age of all diarrhoeal presentations and clinical characteristics of inpatient admissions for diarrhoeal disease

Characteristic	Viral infections	Bacterial infections	Mixed viral/bacterial	Negative	*p*-Value[a]
All diarrhoeal presentations					
Age (months), median (IQR)	7.3 (5.6–9.1)	6.7 (4.6–9.2)	8.0 (6.2–9.4)	5.9 (4.0–7.8)	<0.001
Total count	391	227	133	563	
Total count, Dong Thap	322	207	123	492	
Total count, Ho Chi Minh	69	20	10	71	
Incidence per 1000 IYO	62.7	36.4	21.3	90.2	
Incidence per 1000 IYO, Dong Thap	146.4	94.1	55.9	223.7	
Incidence per 1000 IYO, Ho Chi Minh	17.1	5.0	2.5	17.6	
Inpatient admissions					
Total, *n* (%)	67 (17.1)	22 (9.7)	35 (26.3)	57 (10.1)	<0.001
Incidence per 1000 IYO	10.7	3.5	5.6	9.1	
Vital signs at day 1 of admission					
Maximum temperature, °C	37.5 (37.0–38.5)	37.9 (37.0–39)	37.8 (37.5–38.5)	37.8 (37.0–38.5)	0.748
Maximum heart rate, beats/min	130 (120–130)	127 (120–130)	120 (120–126)	120 (120–130)	0.049
Maximum respiratory rate, breaths/min	36 (32–40)	37 (34–40)	34 (30–38)	36 (32–40)	0.137
Vomiting day 1, *n* (%)	32 (47.8)	8 (36.4)	21 (60.0)	21 (36.8)	0.134
Haematology day 1, median (IQR)					
AST, U/l	78 (51–100)	30 (22–38)	65 (50–181)	89 (86–117)	0.139
ALT, U/l	65 (15–73)	36 (10–62)	36 (19–206)	38 (20–123)	0.729
WBC, $\times 10^9$/l	8.9 (6.6–11.4)	10.3 (7.64–13.7)	9.2 (7.3–13.0)	8.8 (5.6–10.9)	0.268
Neutrophils, %	49.3 (33.3–61.9)	43.0 (27.5–53.9)	53.7 (35.5–59.4)	38 (23.3–49.3)	0.002
Lymphocytes, %	36.3 (25.4–50.8)	38.5 (32.7–50.6)	35.3 (22.6–48.2)	49.5 (34.3–60.7)	0.004
Maximum HCT, %	35 (33–37)	34 (33–36)	37 (34–38)	35 (33–39)	0.094
Platelets, $\times 10^9$/l	338 (271–427)	296 (257–429)	375 (270–437)	319 (240–392)	0.426
Antimicrobial use, *n* (%)	35 (52.2)	19 (86.4)	20 (57.1)	34 (59.6)	0.043
If yes, duration (days), median (IQR)	5 (3–6)	5 (5–6)	5 (4–6)	5 (4–6)	0.624
Admission to ICU, *n* (%)	2 (3.0)	0 (0)	0 (0)	1 (1.8)	0.640
Parenteral fluid administered, *n* (%)	12 (19.4)	2 (9.1)	5 (14.3)	6 (10.5)	0.464
Length of hospital stay (days), median (IQR)	5 (3–6)	4 (3–5)	4 (3–6)	4 (3–6)	0.711

IQR, interquartile range; IYO, infant-years of observation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; WBC, white blood cell count; HCT, haematocrit; ICU, intensive care unit

[a] *p*-Value from Chi-square test or Kruskal–Wallis test, as appropriate.

differ significantly between aetiological groups. Infants admitted to hospital with mixed viral/bacterial infections had a higher proportion of neutrophils (median 54%, IQR 36–59%; Kruskal–Wallis $p = 0.002$) and lower proportion of lymphocytes (median 35.3%, IQR 22.6–48.2%; Kruskal–Wallis $p = 0.004$) than those in the other aetiological groups, including those with negative samples. Infants admitted with bacterial infections were most likely to be prescribed an antimicrobial (86%, 19/22) ($p = 0.043$, Chi-square). The average length of stay in hospital for all admitted diarrhoeal episodes was 5 days (IQR 3–7 days).

3.5. Risk factors for diarrhoeal disease

Risk factors for diarrhoea were investigated by site. In the unadjusted analysis, increased maternal education was protective against diarrhoea in HCMC, whereas male sex, household crowding, use of a piped water supply, and filtering drinking water were all significant risks (Table 3). In a multivariable analysis, maternal education (incidence rate ratio (IRR) 0.75, 95% confidence interval (CI) 0.56–1.00) remained independently associated with protection, and household crowding (≥2 people/room; IRR 1.45, 95% CI 1.07–1.95) along with filtering drinking water (IRR 1.81, 95% CI 1.17–2.81) remained risk factors in this setting.

In Dong Thap, the most important protective factors included maternal age at delivery, maternal education, and filtering of the drinking water supply (Table 4). Male sex and the lack of a flush toilet were risk factors in this setting. After adjusting for confounding, male sex remained the only strongly associated risk factor (IRR 1.20, 95% CI 1.04–1.40), and maternal age (IRR 0.98, 95% CI 0.96–0.99) and education (IRR 0.75, 95% CI 0.62–0.91) remained protective.

3.6. Spatial clustering

As shown in Figure 3, in Dong Thap there was evidence of spatial clustering for each detected pathogen. For all-cause diarrhoea, a cluster was identified with a radius of 6.7 km in the northwest region of the study area (relative risk (RR) 1.79, $p < 0.001$). All of the pathogen-specific clusters centred generally around the same area, in the more rural part of the Dong Thap study area, with radii ranging from 6.6 km (rotavirus) to 12.4 km

Table 3
Risk factors for diarrhoeal presentation in Ho Chi Minh City (HCMC)

Characteristic	IYO	Outpatient		Hospitalized		Total diarrhoea		Unadjusted IRR	Adjusted IRR	*p*-Value
		Cases	CI	Cases	CI	Cases	CI			
Infant-years of follow-up	4040	343	85	18	4	361	89			
Maternal										
Age at delivery	-	-	-	-	-	-	-	1.01 (0.98–1.03)	1.01 (0.98–1.03)	0.555
Education										
Lower secondary or below	2410.7	237	98	12	5	249	103	1.00	1.00	
Higher secondary or above	1629	106	65	6	4	112	69	0.67 (0.51–0.87)[d]	0.75 (0.56–1.00)[d]	0.053
Infant										
Sex										
Female	1895.6	140	74	7	4	147	78	1.00	1.00	
Male	2144.3	203	95	11	5	214	100	1.29 (1.00–1.66)[d]	1.24 (0.96–1.61)	0.105
Low birth weight[a]										
No	3853.5	334	87	16	4	350	91	1.00	1.00	
Yes	186.5	9	48	2	11	11	59	0.66 (0.33–1.31)	0.69 (0.34–1.40)	0.308
Rotavirus vaccine[b]										
No	3018.5	267	88	17	6	284	94	1.00	1.00	
Yes	1021.5	76	74	1	1	77	75	0.81 (0.6–1.09)	0.91 (0.66–1.24)	0.563
Breastfed after birth?										
Yes, exclusively	340.7	30	88	3	9	33	97	1.00	1.00	
Yes, plus formula	3138.7	267	85	11	4	278	89	0.92 (0.59–1.44)	0.87 (0.55–1.37)	0.541
No, only formula	560.6	46	82	4	7	50	89	0.92 (0.54–1.59)	0.87 (0.50–1.53)	0.637
Older sibling										
No	1699.3	130	77	7	4	137	81	1.00	1.00	
Yes	2340.4	213	91	11	5	224	96	1.19 (0.92–1.54)	1.03 (0.77–1.38)	0.838
Household										
Elevation ≤3 m										
No	3086.4	274	89	18	6	292	95	1.00	1.00	
Yes	953.5	69	72	0	0	69	72	0.77 (0.56–1.05)	0.77 (0.56–1.07)	0.116
Household crowding[c]										
No	1298.3	80	62	3	2	83	64	1.00	1.00	
Yes	2587	255	99	14	5	269	104	1.62 (1.21–2.17)[d]	1.45 (1.07–1.95)[d]	0.015
Toilet type										
Own flush	3527.5	303	86	16	5	319	90	1.00	1.00	
Shared flush	493.7	37	75	1	2	38	77	0.85 (0.57–1.28)	0.91 (0.58–1.41)	0.659
Other	18.6	3	161	1	54	4	215	2.37 (0.55–10.27)	1.81 (0.42–7.76)	0.423
Water source										
Piped to home	2711.8	251	93	13	5	264	97	1.38 (1.04–1.85)[d]	1.87 (0.81–4.34)	0.14
Bottled	1223.4	82	67	4	3	86	70	1.00	1.00	
Other	104.6	10	96	1	10	11	105	1.49 (0.68–3.27)	2.14 (0.70–6.53)	0.179
Water treatment										
Boil	2518.4	214	85	9	4	223	89	1.00	1.00	
Filter	269	41	152	4	15	45	167	1.88 (1.22–2.90)[d]	1.81 (1.17–2.81)[d]	0.008
None/other	1252.4	88	70	5	4	93	74	0.84 (0.63–1.12)	1.43 (0.62–3.27)	0.402

IYO, infant-years of observation; CI, cumulative incidence per 1000 infant-years; IRR, incidence rate ratio.
[a] <2500 g.
[b] One or more doses.
[c] Two or more people per room.
[d] Significant at $p = <0.05$.

Table 4
Risk factors for diarrhoeal presentation in Dong Thap

Characteristic	IYO	Outpatient		Hospitalized		Total diarrhoea		Unadjusted IRR	Adjusted IRR	*p*-Value
		Cases	CI	Cases	CI	Cases	CI			
Infant-years of follow-up	2199.4	1203	547	126	57	1329	604			
Maternal										
Age at delivery	-	-	-	-	-	-	-	0.98 (0.97–0.99)[c]	0.98 (0.96–0.99)[c]	0.006
Education										
Lower secondary or below	1693.8	1002	592	106	63	1108	654	1.00	1.00	
Higher secondary or above	503.4	200	397	19	38	219	435	0.67 (0.56–0.79)[c]	0.75 (0.62–0.91)[c]	0.004
Infant										
Sex										
Female	1077.9	564	523	45	42	609	565	1.00	1.00	
Male	1121.5	639	570	81	72	720	642	1.13 (0.99–1.3)[c]	1.20 (1.04–1.40)[c]	0.014
Low birth weight[a]										
No	2098.8	1150	548	123	59	1273	607	1.00	1.00	
Yes	100.6	53	527	3	30	56	557	0.92 (0.66–1.29)	0.91 (0.63–1.31)	0.603
Breastfed after birth?										
Yes, exclusively	1630.9	891	546	99	61	990	607	1.00	1.00	
Yes, plus formula	555.8	302	543	27	49	329	592	0.98 (0.84–1.15)	1.05 (0.88–1.26)	0.546
No, only formula	12.7	10	790	0	0	10	790	1.27 (0.56–2.89)	1.44 (0.60–3.44)	0.411
Older sibling										
No	1168.9	614	525	70	60	684	585	1.00	1.00	
Yes	1028.3	588	572	55	53	643	625	1.07 (0.93–1.22)	1.17 (0.98–1.39)	0.074
Household										
Household crowding[b]										
No	445	233	524	18	40	251	564	1.00	1.00	
Yes	1523.9	817	536	87	57	904	593	1.05 (0.88–1.26)	0.99 (0.82–1.19)	0.926
Toilet type										
Flush	1315.2	658	500	71	54	729	554	1.00	1.00	
None/bush	524	332	634	30	57	362	691	1.24 (1.05–1.46)[c]	1.03 (0.84–1.27)	0.782
Other	357.1	211	591	24	67	235	658	1.19 (0.98–1.43)	1.00 (0.81–1.24)	0.993
Water source										
Piped to home	682.1	345	506	32	47	377	553	1.08 (0.81–1.44)	2.10 (0.68–6.47)	0.198
Piped to public tap	246	119	484	10	41	129	524	1.02 (0.73–1.43)	1.53 (0.49–4.76)	0.463
Bottled	168.2	77	458	9	54	86	511	1.00	1.00	
River/stream	1026.9	613	597	67	65	680	662	1.29 (0.98–1.7)	2.28 (0.74–7.07)	0.153
Other	73	48	658	7	96	55	754	1.46 (0.95–2.26)	2.64 (0.83–8.36)	0.100
Water treatment										
Boil	1499.5	868	579	82	55	950	634	1.00	1.00	
Filter	508.8	237	466	33	65	270	531	0.84 (0.71–0.99)[c]	0.92 (0.76–1.13)	0.423
None	171.5	82	478	10	58	92	537	0.85 (0.65–1.11)	1.85 (0.61–5.57)	0.277
Other	16.5	15	910	0	0	15	910	1.44 (0.71–2.93)	1.56 (0.74–3.25)	0.250
Pigs in household										
No	1972.8	1058	536	113	57	1171	594	1.00	1.00	
Yes	224.4	144	642	12	53	156	695	1.17 (0.94–1.46)	1.06 (0.82–1.36)	0.633
Poultry in household										
No	1498.7	787	525	90	60	877	585	1.00	1.00	
Yes	698.4	415	594	35	50	450	644	1.11 (0.96–1.28)	0.97 (0.82–1.15)	0.710

IYO, infant-years of observation; CI, cumulative incidence per 1000 infant-years; IRR, incidence rate ratio.
[a] <2500 g.
[b] Two or more people per room.
[c] Significant at $p = <0.05$.

(Campylobacter) and RRs from 2.3 (rotavirus) to 3.7 (Shigella). No significant spatial clustering was identified in HCMC (data not shown).

4. Discussion

Diarrhoea remains one of the most common yet preventable conditions affecting the poorest children globally.[1] Through a large, longitudinal birth cohort, a substantial burden of diarrhoeal disease in the first year of life was identified in southern Vietnam, with an estimated minimum incidence of 271/1000 IYO. This is an order of magnitude less than an estimate in infants aged <12 months from the late 1990s in rural Hanoi (3.3/child/year),[7] yet it is higher than the incidence estimated in children under 5 years of age in central Vietnam in 2001–2003 (115/1000 child-years).[3] Differences in disease incidence may have arisen from study design, as the study from rural Hanoi included partially active surveillance. Furthermore, although Dong Thap seemingly had a much higher minimum incidence (604/1000 IYO) than HCMC (89/1000 IYO), the large difference is very likely due to under-ascertainment in HCMC, as the number of healthcare providers in this urban setting is much greater than in semi-rural Dong Thap,[13] and cohort participants therefore had greater opportunity to seek care at non-study clinics.

Viral infections represented the largest burden amongst all diagnosed diarrhoeal presentations in this study, confirming an earlier hospital-based study in HCMC.[6] The distribution of aetiologies between the two sites was comparable, with mixed viral infections identified more frequently in HCMC. This may be confounded by under-ascertainment of hospitalized cases, in particular in HCMC, since hospitalized cases were more likely to be bacterial. Campylobacter was the most frequently detected bacterial pathogen in our cohort, which is in contrast to the recently published Global Enteric Multicenter Study (GEMS), which identified Shigella as the third most common cause of disease, behind rotavirus and Cryptosporidium, in moderate to severe diarrhoea in the first year of life across seven different Asian and African countries.[2] As no control specimens were collected

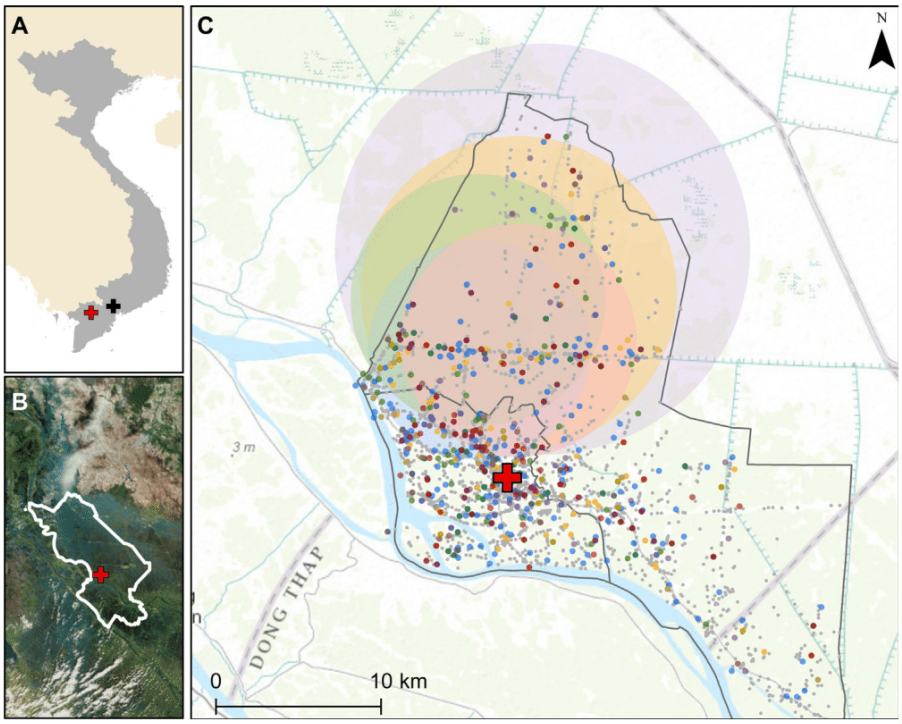

Figure 3. Spatial clustering of diarrhoeal cases in Dong Thap Province. (A) Vietnam is shown in grey, with Ho Chi Minh City (HCMC) indicated with the black cross and Dong Thap indicated with the red cross. (B) Location of Dong Thap Province, with the hospital indicated by the red cross. (C) Locations of households with diarrhoea shown with coloured points, as follows: blue, rotavirus; green, norovirus; red, Shigella; orange, Salmonella; purple, Campylobacter. Grey points show the locations of the households of children in the cohort for whom diarrhoea was not reported during the period of follow-up. Shaded circles indicate the location of the significant clusters, with colours corresponding to the individual pathogens listed above. The red cross shows the location of Dong Thap Provincial Hospital. Grey borders show the borders of Cao Lanh district (large border) and Cao Lanh town (small, embedded border). Actual household locations have been jittered for display on the map.

from healthy children in the present study, the aetiological role of the detected organisms cannot be determined. However, results from a hospital-based study in HCMC suggest that these organisms are not frequently identified in children without diarrhoea, with only 13% of approximately 600 non-diarrhoeal controls positive for an enteric pathogen.[6]

Through this work a large burden of potentially vaccine-preventable rotavirus disease was identified in infants. Over half of all samples with an identified aetiology were positive for rotavirus, with 13% of all rotavirus episodes admitted to hospital. Rotavirus vaccine is available as a 'user pays' product in Vietnam (predominantly the Rotarix monovalent vaccine (GlaxoSmithKline)), but uptake is low due to the prohibitive cost (US$ 70–80) and a lack of vaccine availability in many regions, including Dong Thap. Only 24% of children in HCMC were vaccinated against rotavirus in our cohort. The Vietnamese Ministry of Health has sponsored a locally produced, live-attenuated monovalent rotavirus candidate vaccine, with some success in the early stages of clinical evaluation.[14] Previous work has shown that rotavirus vaccination, if GAVI-subsidized, would be cost-effective in Vietnam,[15] and safe when co-administered within the current expanded programme on immunization (EPI) structure.[16] Furthermore, immune responses (IgA and serum neutralizing antibody) measured against the pentavalent vaccine (RotaTeq) in Vietnamese children were shown in one study to be comparable to those among children in Latin America and Europe.[17] This suggests that rotavirus vaccination in Vietnam may not suffer from the same level of reduced immunogenicity that has been observed to occur with orally administered enteric vaccines in developing countries.[18]

The majority of the identified infections were found to have occurred after 6 months of age, potentially due to the waning of protective maternal antibody and generally high rates of breast-feeding after birth,[19,20] and increased exposure to pathogens with the start of consumption of solid foods. The risk factors for diarrhoeal disease identified through this work, including household crowding, low maternal age, and male sex, are generally consistent with the literature.[4,5] In HCMC, drinking filtered piped water was a significant risk for diarrhoeal disease, although the number of families reporting filtering was relatively low. This may be due to the use of ceramic filters that have pores too large to mechanically prevent viruses from entering the drinking water supply.[21] The absence of a measurable protective effect of rotavirus vaccination in HCMC likely reflects the imperfect case ascertainment, as well as the fact that approximately 50% of diarrhoeal episodes with a known aetiology were associated with pathogens other than rotavirus. The identification of increased spatial risk for diarrhoeal disease in the north-western region of Cao Lanh District in Dong Thap may represent a hotspot of transmission, due potentially to poor sanitation or waste management practices.

The most important limitation in this work was the passive nature of diarrhoeal disease episode detection. Although the staff made every effort to ensure disease episodes were recorded, an unknown number of infants with diarrhoeal disease may have attended clinics other than ours, especially in HCMC, and it is acknowledged that the interpretation of the present results is dependent on this limitation. Therefore, the minimum incidence measurements herein likely underestimate the true burden, particularly in HCMC, and the risk factor and spatial analyses may be biased by misclassification of some infants with undetected diarrhoeal illness. This may also have affected the conclusions on diarrhoeal aetiology, if the distribution of pathogens among episodes from which no specimen was available

differed from those specimens tested. The overall loss to follow-up rate was low, although such bias may also be present and important to consider. Finally, the number of pathogens screened for was limited and may explain the lack of an identified pathogen in almost half of the cases. In particular, screening was not performed for any viruses beyond norovirus and rotavirus, and parasites and diarrhoeagenic *Escherichia coli*, which are known to be prevalent amongst children with diarrhoea in industrializing countries, were not investigated.[2]

Further work to more fully determine the epidemiology of diarrhoeal disease in this setting is warranted, particularly in the face of emerging antimicrobial resistance.[6,22] Active, community-based surveillance of high-risk populations would provide a more accurate estimation of the true extent of the burden. Furthermore, as roughly 40% of diarrhoeal episodes collected in the present cohort study lacked a final diagnosis, investigation into the prevalence of additional pathogens, particularly Cryptosporidium,[2] would help local clinicians to better understand the range of potential aetiologies and corresponding therapies for their patient population. To explore these questions, enrolment into a cohort study of young children aged 1–5 years, as an extension of this birth cohort study, has recently been completed, which includes active surveillance for diarrhoeal disease and diagnosis of viral and bacterial gastrointestinal pathogens.[23] Through this, it will also be possible to explore the relative pathogenicity of isolated organisms as well as distinguish reinfection from long-term carriage, due to the collection of stool from healthy children as well.

In conclusion, the most comprehensive epidemiological description of paediatric diarrhoea in infancy in southern Vietnam, to date, is presented herein. A high burden of diarrhoeal disease in infants under the age of 12 months in both an urban and semi-rural setting is documented, with a large proportion due to vaccine-preventable rotavirus infection. Future efforts to integrate either a GAVI-subsidized or a domestically produced rotavirus vaccine into the national EPI schedule should be pursued.

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Conflict of interest: The authors declare they have no competing interests.

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5.1 Appendix A

Table: Rates of breastfeeding in all infants enrolled in Ho Chi Minh City by month of follow up visit, 2009-2013, n(%)

Breastfed?		**Month of follow up**							
		1	2	3	4	6	9	12	Total
Yes		3675 (88.7)	3337 (81.7)	2941 (73.2)	2553 (63.5)	1610 (41.8)	1427 (36.9)	1230 (31.9)	16,773
	Yes, exclusively	1737 (41.9)	1429 (35)	1184 (29.5)	805 (20)	76 (2)	21 (0.5)	1 (0)	5,253
	Yes, plus formula	1938 (46.8)	1908 (46.7)	1750 (43.6)	1623 (40.4)	209 (5.4)	51 (1.3)	5 (0.1)	7,484
	Yes, plus form and food	0 (0)	0 (0)	4 (0.1)	73 (1.8)	909 (23.6)	1044 (27)	993 (25.8)	3,023
	Yes, plus food	0 (0)	0 (0)	3 (0.1)	52 (1.3)	416 (10.8)	311 (8)	231 (6)	1,013
No		467 (11.3)	749 (18.3)	1075 (26.8)	1468 (36.5)	2241 (58.2)	2445 (63.1)	2620 (68.1)	11065
	No, only formula	464 (11.2)	744 (18.2)	1063 (26.5)	1232 (30.6)	264 (6.9)	87 (2.2)	39 (1)	3,893
	No, food only	1 (0)	0 (0)	0 (0)	3 (0.1)	7 (0.2)	22 (0.6)	16 (0.4)	49
	No, food & form	2 (0)	4 (0.1)	8 (0.2)	138 (3.4)	1773 (46)	2269 (58.6)	2563 (66.6)	6,757
	Other	0 (0)	1 (0)	4 (0.1)	95 (2.4)	197 (5.1)	67 (1.7)	2 (0.1)	366
Total		4142 (100)	4086 (100)	4016 (100)	4021 (100)	3851 (100)	3872 (100)	3850 (100)	27,838

6 RESEARCH PAPER 4: The rising dominance of *Shigella* sonnei: An intercontinental shift in the etiology of bacillary dysentery

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SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLoS Neglected Tropical Diseases		
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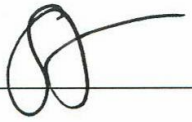
Student Signature: _____ **Date:** 17 Nov 2015

Supervisor Signature:  Date: 17/11/15

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REVIEW

The Rising Dominance of *Shigella sonnei*: An Intercontinental Shift in the Etiology of Bacillary Dysentery

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Abstract

Shigellosis is the major global cause of dysentery. *Shigella sonnei*, which has historically been more commonly isolated in developed countries, is undergoing an unprecedented expansion across industrializing regions in Asia, Latin America, and the Middle East. The precise reasons underpinning the epidemiological distribution of the various *Shigella* species and this global surge in *S. sonnei* are unclear but may be due to three major environmental pressures. First, natural passive immunization with the bacterium *Plesiomonas shigelloides* is hypothesized to protect populations with poor water supplies against *S. sonnei*. Improving the quality of drinking water supplies would, therefore, result in a reduction in *P. shigelloides* exposure and a subsequent reduction in environmental immunization against *S. sonnei*. Secondly, the ubiquitous amoeba species *Acanthamoeba castellanii* has been shown to phagocytize *S. sonnei* efficiently and symbiotically, thus allowing the bacteria access to a protected niche in which to withstand chlorination and other harsh environmental conditions in temperate countries. Finally, *S. sonnei* has emerged from Europe and begun to spread globally only relatively recently. A strong selective pressure from localized antimicrobial use additionally appears to have had a dramatic impact on the evolution of the *S. sonnei* population. We hypothesize that *S. sonnei*, which exhibits an exceptional ability to acquire antimicrobial resistance genes from commensal and pathogenic bacteria, has a competitive advantage over *S. flexneri*, particularly in areas with poorly regulated antimicrobial use. Continuing improvement in the quality of global drinking water supplies alongside the rapid development of antimicrobial resistance predicts the burden and international distribution of *S. sonnei* will only continue to grow. An effective vaccine against *S. sonnei* is overdue and may become one of our only weapons against this increasingly dominant and problematic gastrointestinal pathogen.

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Introduction

Shigellosis, caused by members of the bacterial genus *Shigella*, is a severe and occasionally life-threatening diarrheal infection. Worldwide, *Shigella* spp. are the most common cause of acute,

bloody diarrhea (dysentery) and are responsible for a significant proportion of the burden of morbidity and mortality associated with diarrheal disease [1,2]. In Asia alone, it is estimated that there are 125 million infections and 14,000 deaths due to shigellosis annually [3]. As a result of the considerable global burden, low infectious dose [4], clinical severity, and frequent reports of emerging antimicrobial resistance against first- and, more recently, second-line therapies [5,6], a vaccine against *Shigella* infections is a growing necessity. Yet, more than a century after the discovery of the agent of bacillary dysentery, there is still neither a licensed vaccine nor agreement on the precise mechanisms that induce *Shigella* immunity [7]. Vaccine development is further complicated by the probable need for a multivalent combination of O polysaccharide antigens to protect against a variety of heterogeneously distributed serotypes [8].

The genus *Shigella* incorporates four species. *Shigella dysenteriae* was historically responsible for large epidemics [9] yet is now rarely identified [8]. Similarly, *S. boydii* is also infrequently isolated. *S. flexneri*, however, is common globally and traditionally isolated most frequently in resource-poor countries [10]. *S. flexneri* has 15 different serotypes distributed heterogeneously across different regions, with predominant serotypes including *S. flexneri* 2a, 3a, and 6 [8,11]. Finally, *S. sonnei* is also prevalent globally, although traditionally most commonly detected in high-income regions [10,12]. *S. sonnei* has only one serotype.

S. sonnei: An Emergent Pathogen

Reasons behind the conventional dominance of *S. sonnei* in industrialized countries remain unclear [13]. However, an increasing proportion of shigellosis due to *S. sonnei* generally correlates with improving economic prosperity [12], which in the context of many rapidly developing countries, has led to a proportional decrease in *S. flexneri* and the simultaneous emergence of *S. sonnei* [14]. This shift toward *S. sonnei* has been documented in many regions in Asia, Latin America, and the Middle East (Fig 1) [15–20], with proven explanations behind such an epidemiological phenomenon lacking. This review aims to summarize the existing evidence as to why *S. sonnei* may predominate in high-income countries and why it is now emerging in regions traditionally dominated by *S. flexneri* and explores the implications of the growing threat of this increasingly antimicrobial-resistant pathogen for public health globally.

Plesiomonas shigelloides: Passive Environmental Immunization?

One of the principal theories regarding the lack of *S. sonnei* in industrializing areas focuses on the Gram-negative bacteria *P. shigelloides*, which like *Shigella* falls within the large eubacterial family of the Enterobacteriaece. *P. shigelloides* and *S. sonnei* share an identical lipopolysaccharide (LPS) O-side chain (confirmed by nuclear magnetic resonance [NMR] and mass spectrometry) that is thought to be the major surface antigen targeted by the adaptive immune system during *Shigella* infection [21,22]. These surface antigens are cross-reactive, and vaccines prepared from O-antigen derived from *P. shigelloides* have been shown to be reasonably effective in preventing infection with *S. sonnei* in humans [23,24]. The O-antigen gene cluster is located on the *S. sonnei* invasion plasmid and is essential for penetration of host epithelial cells [25]. Evidence suggests not only that *S. sonnei* acquired the O-antigen gene cluster from *P. shigelloides* but also that this acquisition was the defining event in the emergence of *S. sonnei* [26].

Due to the cross-reactive nature of the *S. sonnei/P. shigelloides* O-antigens, Sack and colleagues suggested that exposure to *P. shigelloides* serotype O17 leads to protection against infection with *S. sonnei* [27]. In areas with poor-quality water supplies, the authors postulated that exposure to *P. shigelloides* occurs frequently and thus disease due to *S. sonnei* is rare, as the population is effectively naturally immunized [27]. Although *P. shigelloides* is found in water

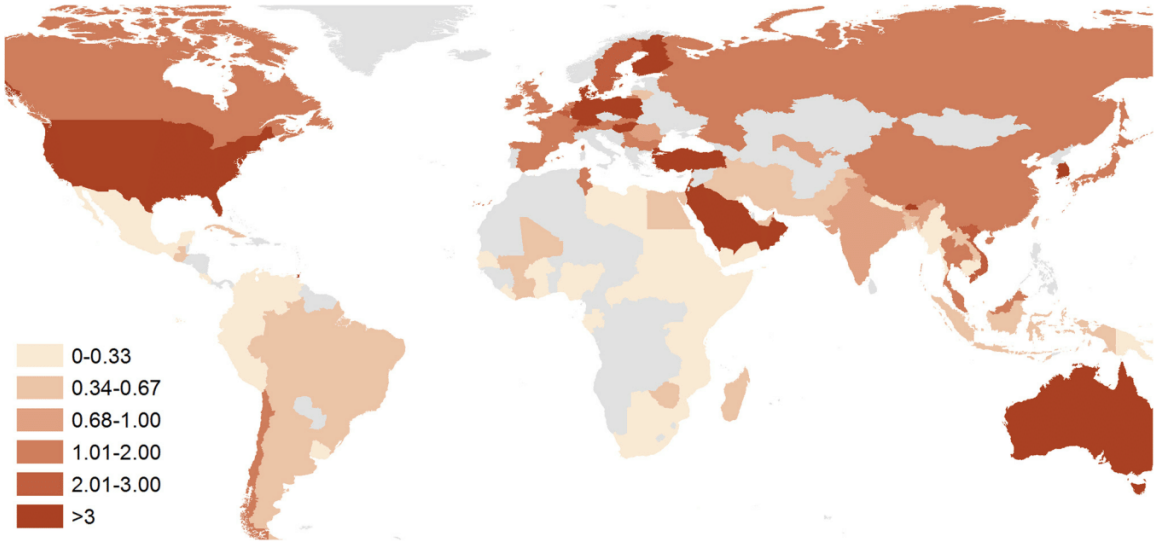

Fig 1. The ratio of *S. sonnei* to *S. flexneri* isolated from 100 countries, 1990–2014. The darker the color, the higher the proportion of *S. sonnei* isolated from each country; the lighter the color, proportionally higher the proportion of *S. flexneri* isolated. Countries colored grey indicate no data on species were identified. To generate this map, we performed an extensive literature review in PubMed using the term "*Shigella*" followed by the name of 178 countries. The most recent publication that included species information that was nonoutbreak and nontravel associated was included as representative of each country. If country data were pre-1990 or were not available on PubMed, the Gideon Infectious Disease encyclopedia as well as national reference laboratory data were referenced where possible. References are listed by country in the supplementary material (S1 Table).

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and environmental samples in both industrialized and industrializing countries [28,29], water treatment practices are likely to prevent frequent exposure in regions with adequate sanitation. Highlighting this, outbreaks of diarrheal disease thought to be due to *P. shigelloides* occurred after a lapse in water chlorination in Japan in the mid-1970s [30]. Although the serotypic distribution of *P. shigelloides* has not been well described [31], serotype O17 has been reported in both water and stool samples from patients admitted for diarrhea in industrializing regions [32,33], lending credence to the hypothesis of water-driven immunization at the population level.

The phenomenon of passive immunization in low-income countries would explain, at least in part, why *S. sonnei* is proportionally more commonly isolated in industrialized countries. Accordingly, an increase in the proportion of *Shigella* episodes due to *S. sonnei* would occur concurrently with economic development and improved water supplies [27]. Ram and colleagues confirmed this economic trend by identifying a strong positive correlation between country-level GDP and proportion of isolated *Shigella* due to *S. sonnei* from 56 studies conducted from 1984–2005 ($R = 0.55$, $p < 0.0001$) [12]. Therefore, the combination of improving economic outlook and fulfillment of the Millennium Development Goals (MDGs) will lead to an improvement of drinking water supply, a drop in population-level cross protection against *S. sonnei*, and potentially to a global increase in *S. sonnei* infections in heavily populated regions currently undergoing such transitions [34].

Acanthamoeba: An Environmental Host?

Acanthamoeba is the most common amoeba found globally, with a wide distribution in both aquatic and nonaquatic environments [35]. Amoeba such as *Acanthamoeba* are known to act as environmental hosts for a variety of intracellular pathogens including *Helicobacter pylori*,

Vibrio cholerae, and also various *Shigella* spp. [36–38]. The uptake of bacteria into amoebic cysts allows the bacteria to persist in adverse environmental conditions, including desiccation, starvation, and a variety of chemical and physical agents [39]. *Acanthamoeba* cysts, which form when triggered by nutritional or osmotic stress, are particularly resistant to chlorine treatment [40]. Found commonly through environmental sampling [41], *Acanthamoeba* has been identified in public water supplies in developed countries with appropriate chlorination levels [42] and in hospital water supplies [43] and can also be isolated from drinking water supplies in industrializing regions [44].

Recent evidence confirms that *S. sonnei*, *S. dysenteriae*, and *S. flexneri* can be taken up by the species *Acanthamoeba castellanii* when grown under laboratory conditions [36,45,46]. Once phagocytized, *Shigella* spp. are localized in *A. castellanii* vacuoles and eventually in the cysts [36,45] and can survive for over three weeks [45]. Notable differences in symbiotic growth were recorded with respect to amoebic uptake between *Shigella* species. *S. sonnei* has been shown to be efficiently taken up and maintained by *A. castellanii* at temperatures between 18–30°C [36,46]; indeed, growth of *S. sonnei* in the presence of *Acanthamoeba* was found to exceed that of *S. sonnei* cultured alone [46]. *S. flexneri*, however, was found to significantly inhibit *A. castellanii* growth in the laboratory at 30°C [46]. Inhibition and killing of *A. castellanii* by *S. flexneri* is due to activation of invasion genes, which may induce apoptosis through the secretion of effector proteins into the host cell via the type three secretion system [47,48].

The growth and survival rates of *Shigella* in the cytoplasm of the amoeba resemble their pattern of growth and survival in mammalian macrophages [45,49]. In fact, it has been suggested that growth in the amoebic intracellular niche may have influenced the ability of *Shigella* to survive in the mammalian phagocytic cell environment [50]. Additionally, as free-living amoeba feed on bacteria, fungi, and algae, lateral gene transfer within the amoeba phagolysosome may have facilitated genetic adaptations that allow for the expression of pathogenic or symbiotic phenotypes based on impact on the host cell [51,52]. It has been further suggested that amoebae themselves represent an important genetic reservoir for internalized microbes [51]. For example, there has been an observed increase in resistance to various antimicrobials and biocides in *Legionella pneumophila* grown within free-living amoeba [53], which may be due to selection within the amoeba itself [51].

All aspects considered, available evidence suggests that *A. castellanii* may contribute to transmission of *S. sonnei* in temperate regions by phagocytizing *S. sonnei*, thus allowing the bacteria to circumvent the effects of chlorination and good sanitation [54]. As *S. flexneri* has been shown to inhibit growth of *A. castellani* [46], the amoeba may not be a viable reservoir for *S. flexneri* in either developed or industrializing countries. Protozoa appear to play an important role in the transition of bacteria from the environment to mammals and as such may be the source of emerging pathogenic bacteria [50], and may play an increasing role in the epidemiology of *S. sonnei* in industrializing regions as the prevalence increases.

Antimicrobial Resistance: A New Defense Strategy?

Phylogeographical analyses of a large number of *S. sonnei* isolates spanning several continents and several decades in a publication by Holt et al. demonstrated that all contemporary *S. sonnei* infections are due to a small number of clones that dispersed globally from Europe within the last 500 years [34]. Four distinct lineages of *S. sonnei* were identified, with lineage III the most prevalent globally, becoming dominant in Asia, Africa, and South America [34]. *S. sonnei* belonging to lineage III are characterized by the presence of distinct class II integron (In*2*), which confers resistance to trimethoprim, streptothricin, and streptomycin [55]. Many lineage III isolates were also found to harbor a genetic locus on a small plasmid conferring resistance to a

variety of additional antimicrobials including tetracycline and sulphonamides. Holt and colleagues indicated that In*2* was likely acquired during the mid-20th century, after which the clone spread internationally, undergoing contemporary global dispersal and localized clonal expansions [34].

This localized microevolution of *S. sonnei* appears to be largely driven by selection pressure induced by antimicrobials [56]. The determinants for antimicrobial resistance in *Shigella* are generally located on mobile genetic elements such as plasmids, transposons, and integrons [55]. Horizontal gene transfer (HGT) of such elements is now recognized to be an important driver of bacterial evolution [57,58]. One study, for example, estimated 18% of the 4,288 genes of *Escherichia coli* strain MG1655 were acquired laterally since the species diverged from the *Salmonella* lineage 100 million years (Myr) ago, a rate of 16 kb/Myr/lineage [59]. Transfer of mobile genetic elements between members of the Enterobacteriaceae is known to be responsible for the dissemination of antimicrobial resistance genes and the emergence of a variety of multidrug-resistant (MDR) Gram-negative bacteria globally [55,60,61].

S. sonnei can acquire advantageous chromosomal and plasmid-mediated resistance genes through HGT from both commensal and pathogenic bacteria circulating locally, enhancing its ability to establish infection, prolonging shedding, and, presumably, outcompeting antimicrobial-susceptible bacteria [56]. A study of >250 *S. sonnei* isolates collected over 15 years in Vietnam documented the rapid emergence and dominance of successful clones of *S. sonnei* after the acquisition and fixation of plasmids conferring colicin production/immunity and resistance to third-generation cephalosporins in two separate genetic bottleneck events [56]. In areas with unregulated antimicrobial use, *S. sonnei* may have abundant opportunity to acquire locally derived resistance genes [62]. In countries with restricted antimicrobial usage, for example, *S. sonnei* are generally more susceptible to quinolones [5], presumably because of lower selective pressure combined with reduced availability of resistance genes in the circulating accessory gene pool. Such a phenomenon is thought to be leading to increasingly successful clones in areas of unregulated antimicrobial use and could lead to a rapidly growing and increasingly challenging public health problem in many industrializing areas [56].

In 2005, the WHO published guidelines recommending ciprofloxacin to be used as the first-line treatment for dysentery [63]. The late 2000s saw the first documented resistance in *S. sonnei* against fluoroquinolones [5]. Phylogeographical data from Holt and colleagues indicate marked differences in the global prevalence of *gyrA* (DNA gyrase) mutations, which confer resistance to quinolones and reduced susceptibility to fluoroquinolones. Strong selection for quinolone resistance was identified, as the facilitating mutations have occurred independently on multiple occasions in several different lineages and genetic locations [34]. Unless the use of fluoroquinolones becomes regulated in areas of current unrestricted use, ciprofloxacin will likely become ineffective for treating *Shigella* infections in the near future [5]. Yet pivmecillinam, ceftriaxone, or azithromycin may be effective alternatives [64,65], depending on local resistance patterns.

Does *Shigella sonnei* Have a Competitive Advantage?

Taken together, evidence suggests that the global burden of *S. sonnei* may be growing compared to that of *S. flexneri*. This phenomenon may not only be due to the global improvements in water quality and an ability of *S. sonnei* to grow successfully within *Acanthamoeba* but may also be due to a potential, but as yet unproven, ability to acquire and/or maintain a wider array of antimicrobial resistance genes. Indeed, it has been speculated that the plasmid composition and resistance profiles may differ between the *Shigella* species isolated from contemporaneous patient populations in the same locations (Fig 2) [15,66–71]. Although *S. sonnei* can acquire

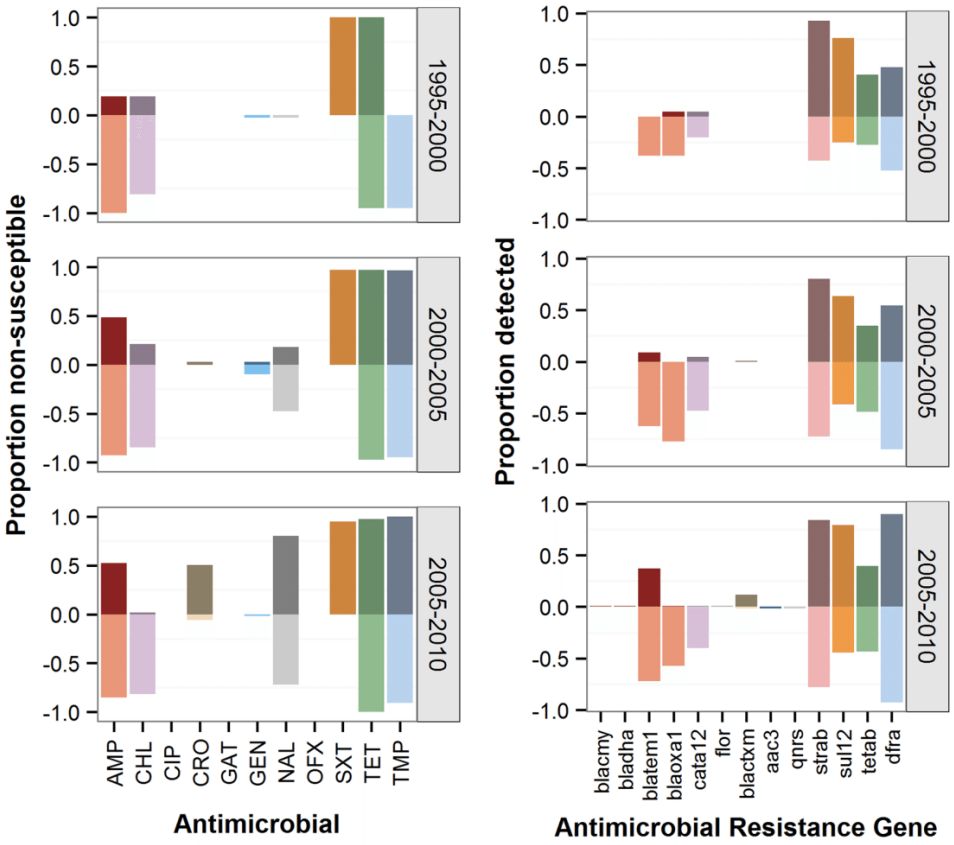

Fig 2. Antimicrobial resistance and presence of resistance-conferring genes in *S. sonnei* and *S. flexneri*. Plots on the left show proportion of antimicrobial resistance determined by minimum inhibitory concentration (MIC) amongst isolates collected from Vietnam over a 15-year period ($n = 231$ for *S. sonnei* and 136 for *S. flexneri*) [34,56]. *S. sonnei* are shown by darker colors on the top of each graph, and *S. flexneri* is shown by lighter colors at the bottom. Plots on the right show the proportion of *S. sonnei* (dark color, top of each graph) and *S. flexneri* (light color, bottom of each graph) found to have varying resistance genes present on either plasmids or the chromosome. See Supporting Information (SI) S1 Text for a description of procedures. The color of the gene corresponds with the color of the antimicrobial to which it confers resistance on the left. AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; CRO: ceftriaxone; GAT: gatifloxacin; GEN: gentamicin; NAL: nalidixic acid; OFX: ofloxacin; SXT: cotrimoxazole; TET: tetracycline; TMP: trimethoprim.

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extended-spectrum beta-lactamase (ESBL)-mediated resistance from other Enterobacteriaceae, particularly *E. coli* and *Klebsiella* spp. [72], it is not currently known whether *S. flexneri* and *S. sonnei* acquire resistance genes from each other. Toro et al. suggested that there is a greater restriction barrier for conjugal plasmids between *S. sonnei* and *S. flexneri* than between other Gram-negative donors and recipients [67]. A differential ability to acquire and/or maintain plasmids between *S. flexneri* and *S. sonnei* from other bacterial donors may also explain discrepant resistance profiles between contemporaneous species, although such a phenomenon has yet to be explicitly investigated.

Levels of inflammation in the gut during infection could explain differences in the ability to acquire mobile genetic elements of resistance between the species. Stecher et al. demonstrated that inflammatory responses in the gut during infection may facilitate conjugative transfer and reassortment of plasmid-encoding genes between pathogens and commensal organisms [73]. Although it was shown recently that *S. flexneri* is able to modify its LPS structure to dampen the inflammatory innate immune response to allow it to successfully evade detection in the

initial phases of infection [74], it is not yet known whether this occurs during *S. sonnei* infection. Investigations into differential inflammatory response between the *Shigella* species during infection and their relationship to HGT within the human gastrointestinal tract are warranted.

Finally, *S. sonnei* has been shown to be genetically more similar to its ancestor *E. coli* than to other *Shigella* species [75,76]. Gene retention from *E. coli* potentially imbues *S. sonnei* with a higher likelihood for survival in the environment or an environmental adaptive host [75], such as *Acanthamoeba*. *S. flexneri*, however, loses genes faster than any other *Shigella* species and is the most genetically distant of the *Shigellae* from *E. coli* [76]. Hershberg and colleagues suggest a potential "point of no return" for *Shigella* in that once it undergoes enough purifying selection, it cannot regain enough of its lost functionality to escape niche limitation [76]. Does enhanced capacity for genomic plasticity explain the hypothesized increased ability of *S. sonnei* to acquire or maintain plasmids from other bacterial donors [77]? Experimental evidence of differences in gene acquisition and retention between the two species is needed.

Next Steps

We predict that the combination of improving water supplies and rapid acquisition and maintenance of mobile elements conferring advantageous resistance genes is accelerating a *Shigella* species shift toward *S. sonnei* dominance, which traditionally has been shown to occur over a period of decades in individual countries (Table 1, S1 Table) [78,79]. To counter this rapid species replacement, many questions regarding the epidemiology of *S. sonnei* and, crucially, vaccine development need to be addressed. Identifying prominent transmission routes of *S. sonnei* and *S. flexneri* in resource-poor countries should remain a primary goal. Indeed, experiments to determine the relative fitness of each species in varying environmental conditions and investigating antimicrobial fitness [80] would provide information on potential niche preferences and add insight into which accessory gene pools each species samples from. Finally, longitudinal monitoring of water supplies for the presence of both *P. shigelloides* and *A. castellanii* would help to verify the hypotheses presented in this review in regard to both a reduction of population immunity against the *S. sonnei* O-antigen as well as an environmental amoeba niche for these bacteria.

Furthermore, research on the genetic structure of global *S. flexneri* populations is warranted in order to help further understand the global species shift and to explore the global and

Table 1. Summary of factors behind the traditional and current epidemiological distribution of *S. sonnei* and *S. flexneri*.

Factor	Region	
	Industrializing	Industrialized
Explanation of traditional geographical distribution	1. *S. sonnei* is not present because of population immunity due to cross protection from exposure to *P. shigelloides* found in contaminated water supplies [26]	1. *S. sonnei* is present because of a lack of cross protection from exposure to *P. shigelloides* due to clean water supplies
	2. *S. flexneri* is not able to grow within the common amoeba *A. castellanii* [46]	2. *S. sonnei* is symbiotically phagocytosed by *A. castellanii* and can withstand chlorination and other harsh environmental conditions [36]
Why is the burden of *S. sonnei* growing?	1. Improving water supplies may lead to a decrease in the prevalence of *P. shigelloides*, resulting in lack of cross protection against *S. sonnei* [27]	
	2. Expansion of *S. sonnei* from Europe in the last 500 years and subsequent microevolution due largely to local antimicrobial use [34,56]	
	3. Proposed competitive advantage of *S. sonnei* against *S. flexneri* due to an enhanced ability to acquire and maintain mobile resistance genes from other bacterial species	

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localized microevolution of this pathogen over time. Such analyses would help to predict the role of *S. flexneri* in the context of improving sanitation and growing prevalence of *S. sonnei* worldwide. Finally, although plagued with many setbacks [11], the development of a sufficiently safe and effective *S. sonnei* vaccine may be feasible in the coming decade. However, in order to carry out properly designed vaccine trials in the future, outstanding questions regarding correlates of immunity, incidence in the community, seroconversion rates, and the role of maternal antibody in the first years of life will need to be answered.

In conclusion, *S. sonnei* represents an emerging threat to public health globally. With continuing efforts for improvements in water and sanitation worldwide, population-level immunization against *S. sonnei* due to exposure to *P. shigelloides* is declining. Additionally, environmental hosts such as *A. castellani* represent an important yet potentially overlooked reservoir of *S. sonnei* and may explain in part the persistence of *S. sonnei* in regions with a reasonably good standard of sanitation. Finally, the incredible ability of *S. sonnei* to acquire resistance to a variety of widely used antimicrobials may endow the pathogen with a competitive advantage over sensitive bacterial competitors and predicts its emergence in areas with unregulated antimicrobial use. Combined, this evidence suggests alarming increases in global prevalence of *S. sonnei* and unprecedented levels of resistance, demanding a vaccine in the near future that can be administered to the most vulnerable populations, particularly young children in rapidly industrializing countries.

Boxes

Box 1. Key Learning Points

1. Traditionally, the various species of the bacterial genus *Shigella* have a distinct geographical distribution. *S. sonnei* is most commonly isolated in industrialized countries, whereas *S. flexneri* is more commonly isolated in industrializing regions. However, *S. sonnei* is now becoming recognized as a common enteric pathogen in many industrializing regions. The exact mechanisms catalyzing this shift in the epidemiological distribution are unclear.
2. Improving the quality of drinking water supplies in industrializing regions is likely to reduce cross protection against *S. sonnei* derived from the bacterium *P. shigelloides*, which is commonly found in contaminated water.
3. *S. sonnei* may be efficiently phagocytized by the ubiquitous amoeba species *A. castellani*, thereby providing it with a reservoir in which to withstand chlorination and other harsh environmental conditions.
4. In comparison to *S. flexneri*, *S. sonnei* has a greater ability to develop resistance to broad-spectrum antimicrobials. We suggest that *S. sonnei* is more likely to accept and maintain horizontally transferred DNA, which gives it a competitive advantage against *S. flexneri*, particularly in areas with unregulated antimicrobial use.
5. With ongoing improvements in the international quality of water supplies and rapid development of antimicrobial resistance, the burden of *S. sonnei* is likely to grow substantially. A vaccine against *S. sonnei* is increasingly necessary.

Box 2. Top Five Papers

1. Sack D, Hoque A, Huq A, Etheridge M (1994) Is Protection against Shigellosis Induced by Natural Infection with *Plesiomonas shigelloides*? Lancet 343: 1413–1415.
2. Saeed A, Johansson D, Sandström G, Abd H (2012) Temperature Depended Role of *Shigella flexneri* Invasion Plasmid on the Interaction with *Acanthamoeba castellanii*. Int J Microbiol 2012: 917031.
3. Holt KE, Baker S, Weill F-X, Holmes EC, Kitchen A, et al. (2012) *Shigella sonnei* Genome Sequencing and Phylogenetic Analysis Indicate Recent Global Dissemination from Europe. Nat Genet 44: 1056–1059.
4. Holt KE, Thieu Nga TV, Thanh DP, Vinh H, Kim DW, et al. (2013) Tracking the Establishment of Local Endemic Populations of an Emergent Enteric Pathogen. Proc Natl Acad Sci 110: 17522–17527.
5. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB (2007) Clinical Trials of *Shigella* Vaccines: Two Steps Forward and One Step Back on a Long, Hard Road. Nat Rev Microbiol 5: 540–553.

Supporting Information

S1 Table. Country-specific references for Fig 1 showing the ratio of *S. sonnei* to *S. flexneri* isolated from 100 countries, 1990–2014.
(DOCX)

S1 Text. Methods for antimicrobial resistance and gene content data shown in Fig 2. Data are derived from analyses of 367 *Shigella* isolates collected between 1995 and 2010 from Vietnam (136 *S. flexneri* and 231 *S. sonnei*). Resistance was determined by MIC and gene content analysis from Illumina genome sequencing data.
(DOCX)

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6.1 Supplementary Information

Table S1. Country-specific references for figure 1 showing the ratio of *S. sonnei* to *S. flexneri* isolated from 100 countries, 1990-2014.

Listed in alphabetical order by country. Missing countries did not have any publically available data >1990.

Country	Reference
Argentina	Rolfo F, Marin GH, Silberman M, Pattin J, Gatti B, et al. (2012) Epidemiological study of shigellosis in an urban area of Argentina. J Infect Dev Ctries 6: 324–328.
Australia	Australia's Notifiable Disease Status, 2009: Annual Report of the National Notifiable Diseases Surveillance System (2009). Canberra, Australia. Available at http://www.health.gov.au/internet/main/publishing.nsf/ Content/cda-pubs-annlrpt-nndssar.htm
Austria	Bundesministerium Fur Gesundheit: Mitteilungen fur das offentliche Gesundheitswesen (Public Health Newsletter), Ausgabe 3, Quartal 2014 (2014). Wien, Austria. Available at http://bmg.gv.at/cms/home/attachments /0/2/1/CH1184/CMS1412325626202/nl_3_quartal_2014_neuerliche_aussendung_23102014.pdf
Bahrain	Jamsheer A, Bindayna K, Al-Balooshi N, Botta G (2003) Trend of antibiotic resistance in 1316 *Shigella* strains isolated in Bahrain. Saudi Med J 24: 424–426.
Bangladesh	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Belgium	Vrints M, Mairiaux E, Van Meervenne E, Collard J-M, Bertrand S (2009) Surveillance of antibiotic susceptibility patterns among *Shigella sonnei* strains isolated in Belgium during the 18-year period 1990 to 2007. J Clin Microbiol 47: 1379–1385.
Bhutan	Ruekit S, Wangchuk S, Dorji T, Tshering KP, Pootong P, et al. (2014) Molecular characterization and PCR-based replicon typing of multidrug resistant *Shigella sonnei* isolates from an outbreak in Thimphu, Bhutan. BMC Res Notes 7: 95–104.

Bolivia	Townes JM, Quick R, Gonzales OY, Linares M, Damiani E, et al. (1997) Etiology of Bloody Diarrhea in Bolivian Children: Implications for Empiric Therapy. J Infect Dis 175: 1527–1530.
Botswana	Rowe JS, Shah SS, Motlhagodi S, Bafana M, Tawanana E, et al. (2010) An epidemiologic review of enteropathogens in Gaborone, Botswana: shifting patterns of resistance in an HIV endemic region. PLoS One 5: e10924.
Brazil	Da Cruz CBN, de Souza MCS, Serra PT, Santos I, Balieiro A, et al. (2014) Virulence factors associated with pediatric shigellosis in Brazilian Amazon. Biomed Res Int 2014: 539697.
Bulgaria	Bratoeva MP, John JF, Barg NL (1992) Molecular Epidemiology of Trimethoprim-Resistant *Shigella boydii* Serotype 2 Strains from Bulgaria. J Clin Microbiol 30: 1428–1431.
Burkina Faso	Bonkoungou IJO, Haukka K, Österblad M, Hakanen AJ, Traoré AS, et al. (2013) Bacterial and viral etiology of childhood diarrhea in Ouagadougou, Burkina Faso. BMC Pediatr 13: 36–42.
Cambodia	Study: Meng CY, Smith BL, Bodhidatta L, Richard SA, Vansith K, et al. (2011) Etiology of Diarrhea in Young Children and Patterns of Antibiotic Resistance in Cambodia. Pediatr Infect Dis J 30: 331–335. Species data: Dr Ladaporn Bodhidatta, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, Department of Enteric Diseases.
Canada	Canadian Integrated Surveillance Report: Salmonella, Campylobacter, verotoxigenic E. coli and *Shigella*, from 2000-2004 (2009). Available at http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/09vol35/35s3/index-eng.php
Central African Republic	Bercion R, Njuimo SP, Boudjeka PM, Manirakiza A (2008) Distribution and antibiotic susceptibility of *Shigella* isolates in Bangui, Central African Republic. Trop Med Int Heal 13: 468–471.
Chile	Hamilton-West C, J VP, Carlos J, Hormazábal O, Z RL, et al. (2007) Epidemiología clínica y molecular de las infecciones por *Shigella* spp en ninos de la Region Metropolitana durante el verano 2004-2005. Rev Med Chil 135: 1388–1396.
China	Zhang J, Wang F, Jin H, Hu J, Yuan Z, et al. (2014) Laboratory monitoring of bacterial gastroenteric pathogens *Salmonella* and *Shigella* in Shanghai, China 2006-2012. Epidemiol Infect: 1–8.
Colombia	Urbina D, Arzuza O, Young G, Parra E, Castro R, et al. (2003) Rotavirus type A and other enteric pathogens in stool samples from children with acute diarrhea on the Colombian northern coast. Int Microbiol 6: 27–32.

Costa Rica	Achi R, Mata L, Siles X, Lindberg A (1996) Immunomagnetic Separation and PCR Detection Show *Shigellae* to be Common Faecal Agents in Children From Urban Marginal Communities of Costa Rica. J Infect 32: 211–218.
Côte d'Ivoire	Antoine B, Adjehi D, Nathalie G, Valerie G, Etienne D, et al. (2010) Virulence Factors and Resistance Profile of *Shigella* Isolated During Infectious Diarrhea in Abidjan, Côte D' Ivoire. J Appl Sci Res 6: 594–599.
Cuba	Bravo LF, Correa Y, Clausell JF, Fernandez A, Ramírez M, et al. (2009) Caracterización de factores de virulencia y susceptibilidad antimicrobiana en cepas de *Plesiomonas shigelloides* aisladas de pacientes con diarrea aguda en Cuba. Microbiol Clin 26: 233–238.
Denmark	Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon Informatics.
Djibouti	Mikhail IA, Fox E, Haberberger L, Ahmed MH, Abbatte EA (1990) Epidemiology of Bacterial Pathogens Associated with Infectious Diarrhea in Djibouti. J Clin Microbiol 28: 956–961.
Ecuador	Sempertegui F, Estrella B, Egas J, Carrion P, Yerovi L, et al. (1995) Risk of diarrheal disease in Ecuadorian day-care centers. Pediatr Infect Dis J 14: 606–612.
Egypt	Ahmed S, Riddle M, Wierzba R, Messih IA, Monteville M, et al. (2006) Epidemiology and genetic characterization of *Shigella flexneri* strains isolated from three paediatric populations in Egypt (2000-2004). Epidemiol Infect 134: 1237–1248.
Eritrea	Naik D (2006) Prevalence and antimicrobial susceptibility patterns of *Shigella* species in Asmara, Eritrea, northeast Africa. J Microbiol Immunol Infect 39: 392–395.
Ethiopia	Asrat D (2008) *Shigella* and *Salmonella* serogroups and their antibiotic susceptibility patterns in Ethiopia. Le Rev Sante la Mediterr Orient 14: 760–767.
Fiji	Watson C (2006) Death from Multi-resistant shigelloses: a case study from Fiji. Pacific Public Heal 13: 111–114.
Finland	Haukka K, Siitonen A (2008) Emerging resistance to newer antimicrobial agents among *Shigella* isolated from Finnish foreign travellers. Epidemiol Infect 136: 476–482.
France	Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon Informatics.
Gabon	Schaumburg F, Alabi AS, Kaba H, Lell B, Becker K, et al. (2014) Molecular characterization of *Shigella* spp. from patients in Gabon 2011 – 2013. Trans R Soc Trop Med Hyg Nov 20: pii:tru175.
Germany	Robert Koch Institute: Shigellosis (2013). Robert Koch Inst SurvStat@RKI. Available at http://www3.rki.de/SurvStat/QueryForm.aspx.

Ghana	Opintan JA, Newman MJ, Ayeh-Kumi PF, Affrim R, Gepi-Attee R, et al. (2010) Pediatric diarrhea in southern Ghana: etiology and association with intestinal inflammation and malnutrition. Am J Trop Med Hyg 83: 936–943.
Guatemala	Cruz JR, Cano F, Bartlett A V, Mendez H (1994) Infection, diarrhea, and dysentery caused by *Shigella* species and Campylobacter jejuni among Guatemalan rural children. Pediatr Infect Dis J 13: 216–223.
Hungary	Nogrady N, Kiraly M, Borbas K, Toth A, Paszti J, et al. (2013) Antimicrobial resistance and genetic characteristics of integron-carrier shigellae isolated in Hungary. J Med Microbiol 62: 1545–1551.
India	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Indonesia	Herwana E, Surjawidjaja JE, Salim OC, Indriani N, Bukitwetan P, et al. (2010) *Shigella*-associated diarrhea in children in South Jakarta, Indonesia. Southeast Asian J Trop Med Public Health 41: 418–425.
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Iraq	Mohammed AMN (2009) Shigellae – associated diarrhoea in children in Baghdad – Iraq. Iraqi J Med Sci 7: 59–65.
Ireland	Annual Report 2012: Health Protection Surveillance Centre (2012). Dublin. Available at http://www.hpsc.ie/AboutHPSC/AnnualReports/File,14421,en.pdf
Israel	Cohen D, Bassal R, Goren S, Rouach T, Taran D, et al. (2014) Recent trends in the epidemiology of shigellosis in Israel. Epidemiol Infect 142: 2583–2594.
Japan	Infectious Disease Surveillance Center (IDSC) of Japan: *Shigella* (2012). Available at http://idsc.nih.go.jp/.
Jordan	Gargouri N, Walke H, Belbeisi A, Hadadin A, Salah S, et al. (2009) Estimated Burden of Human *Salmonella*, *Shigella*, and Brucella Infections in Jordan, 2003–2004. Foodborne Pathog Dis 6: 481–487.
Kenya	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Kuwait	Jamal W, Rotimi VO, Pal T, Sonnevend A, Dimitrov TS (2010) Comparative in vitro activity of tigecycline and other antimicrobial agents against *Shigella* species from Kuwait and the United Arab of Emirates. J Infect Public Health 3: 35–42.
Laos	Phetsouvanh R, Midorikawa Y, Nakamura S (1999) The seasonal variation in the microbial agents implicated in the etiology of diarrheal diseases among children in Lao People's Democratic Republic. Southeast Asian J Trop Med Public Health 30: 319–324.

Lebanon	Araj GF, Avedissian AZ, Ayyash NS, Bey HA, Asmar RG El, et al. (2012) A reflection on bacterial resistance to antimicrobial agents at a major tertiary care center in Lebanon over a decade. Leban Med J 60: 125–135.
Liberia	Guyot A (1996) Antibiotic resistance in *Shigella* in Monrovia. Trop Doct 26: 70–71.
Libya	Ali MB, Ghenghesh KS, Aissa RB, Abuhelfaia A, Dufani M (2005) Etiology of childhood diarrhea in Zliten, Libya. Saudi Med J 26: 1759–1765.
Lithuania	Jensen G, Wandall D, Gaarslev K, Panavas S, Gutschik E (1996) Antibiotic Resistance in *Shigella* and *Salmonella* in a Region of Lithuania. Eur J Clin Microbiol Infect Dis 15: 872–876.
Madagascar	Randrianirina F, Ratsima EH, Ramparany L, Randremanana R, Rakotonirina HC, et al. (2014) Antimicrobial resistance of bacterial enteropathogens isolated from stools in Madagascar. BMC Infect Dis 14: 104.
Malawi	Pitman C, Amali R, Kanyerere H, Siyasiya A, Phiri S, et al. (1996) Bloody diarrhoea of adults in Malawi: antimicrobial sensitivities clinical features, infectious agents, and antimicrobial sensitivities. Trans R Soc Trop Med Hyg 90: 284–287.
Malaysia	Banga Singh K-K, Ojha SC, Deris ZZ, Rahman RA (2011) A 9-year study of shigellosis in Northeast Malaysia: Antimicrobial susceptibility and shifting species dominance. Zeitschrift fur Gesundheitswissenschaften 19: 231–236.
Mali	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Mexico	Zaidi MB, Estrada-García T, Campos FD, Chim R, Arjona F, et al. (2013) Incidence, clinical presentation, and antimicrobial resistance trends in *Salmonella* and *Shigella* infections from children in Yucatan, Mexico. Front Microbiol 4: 288.
Montenegro	Sipetic-Grujicic S, Glusac S, Ratkov I, Maksimovic J, Ratkov E, et al. (2010) Shigellosis - epidemiological situation in Montenegro in period 1996-2005 [Serbian]. Med Pregl 63: 554–557.
Mozambique	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Myanmar	Oo K, M T (1995) Serotype distribution and antimicrobial susceptibility of *Shigellae* isolated from diarrhoeal patients in Yangon, Myanmar. J Diarrhoeal Dis Res 13: 180–182.
Nepal	Kansakar P, Baral P, Malla S, Ghimire GR (2004) Antimicrobial susceptibilities of enteric bacterial pathogens isolated in Kathmandu, Nepal, during 2002-2004. J Infect Dev Ctries 5: 163–168.
Netherlands	Van Pelt W, de Wit M, Wannet W, Ligtvoet E, Widdowson M, et al.

	(2003) Laboratory surveillance of bacterial gastroenteric pathogens in The Netherlands, 1991 – 2001. Epidemiol Infect 130: 431–441.
Nigeria	Abdu A, Aboderin AO, Elusiyan JB, Kolawole D, Lamikanra A (2013) Serogroup distribution of *Shigella* in Ile-Ife, southwest Nigeria. Trop Gastroenterol 34: 164–169.
Oman	Patel P, Mercy J, Shenoy J, Ashwini B (2008) Factors associated with acute diarrhoea in children in Dhahira, Oman: a hospital-based study. East Mediterr Heal J 14: 571–578.
Pakistan	Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.
Papua New Guinea	Greenhill AR, Guwada C, Siba V, Michael A, Yoannes M, et al. (2014) Antibiotic resistant *Shigella* is a major cause of diarrhoea in the Highlands of Papua New Guinea. J Infect Dev Ctries 8: 1391–1397.
Paraguay	Basualdo W, Arbo A (2003) Randomized comparison of azithromycin versus cefixime for treatment of shigellosis in children. Pediatr Infect Dis J 22: 374–377.
Peru	Kosek M, Yori PP, Pan WK, Olortegui MP, Gilman RH, et al. (2013) Epidemiology of Highly Endemic Multiply Antibiotic-Resistant Shigellosis in Children in the Peruvian Amazon. Pediatrics 122: e541–549.
Poland	Stypulkowska-Misiurewicz H, Baumann-Popczyk A (2013) Shigellosis in Poland in 2011. Przegl Epidemiol 67: 217–219.
Romania	Luca C, Nemescu R, Teodor A, Fantanaru R, Petrovici C, et al. (2011) Etiological aspects of acute gastroenteritis - a ten year review. Rev Med Chir Soc Med Nat Iasi 115: 712–717.
Russia	Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon Informatics.
Rwanda	Bogaerts J, Verhaegen J, Munyabikali JP, Mukantabana B, Lemmens P, et al. (1997) Antimicrobial Resistance and Serotypes of *Shigella* Isolates in Kigali, Rwanda (1983 to 1993): Increasing Frequency of Multiple Resistance. Bacteriology 28: 165–171.
Saudi Arabia	Panhotra BR, Saxena AK, Al-Mulhim K (2004) Emergence of Nalidixic Acid Resistance in *Shigella sonnei* Isolated from Patients Having Acute Diarrheal Disease: Report from Eastern Province of Saudi Arabia. Japanese J Infecious Dis 57: 116–118.
Senegal	Sire J-M, Garin B, Chartier L, Fall NK, Tall A, et al. (2013) Community-acquired infectious diarrhoea in children under 5 years of age in Dakar, Senegal. Paediatr Int Child Health 33: 139–144.
Serbia	Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon Informatics.
Slovakia	Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon

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Somalia — Casalino M, Nicoletti M, Salvia A, Colonna B, Pazzani C, et al. (1994) Characterization of Endemic *Shigella flexneri* Strains in Somalia: Antimicrobial Resistance, Plasmid Profiles, and Serotype Correlation. J Clin Microbiol 32: 1179–1183.

South Africa — Tau NP, Smith AM, Sooka A, Keddy KH (2009) Molecular characterization of extended-spectrum beta-lactamase-producing *Shigella* isolates from humans in South Africa, 2003-2009. J Med Microbiol 61: 162–164.

South Korea — Lee JC, Jeong YS, Oh JY, Kang HY, Kim KH, et al. (2006) Epidemiology of Shigellosis in Korea. J Bacteriol Virol 36: 41–49.

Spain — Hernández AC, Calvo AV, Lobato ES, Jiménez FF (2008) Infección por *Shigella* spp. en el Hospital de Getafe entre 2001 y 2006. An Pediatr 68: 605–609.

Sudan — Ahmed AA, Osman H, Mansour AM, Musa HA, Ahmed AB, et al. (2000) Antimicrobial agent resistance in bacterial isolates from patients with diarrhea and urinary tract infection in the Sudan. Am J Trop Med Hyg 63: 259–263.

Sweden — Svenungsson B, Lagergren A, Ekwall E, Evengard B, Hedlund KO, et al. (2000) Enteropathogens in Adult Patients with Diarrhea and Healthy Control Subjects: A 1-Year Prospective Study in a Swedish Clinic for Infectious Diseases. Clin Infect Dis 30: 770–778.

Switzerland — Berger S (2014) Shigellosis: Global Status - 2014 edition. Gideon Informatics.

Taiwan — Wu C-H, Huang L-T, Huang I-F, Liu J-W, Chen J-B, et al. (2009) Acute non-outbreak shigellosis: ten years experience in southern Taiwan. Chang Gung Med J 32: 59–65.

Tanzania — Moyo SJ, Gro N, Matee MI, Kitundu J, Myrmel H, et al. (2011) Age specific aetiological agents of diarrhoea in hospitalized children aged less than five years in Dar es Salaam, Tanzania. BMC Pediatr 11: 19–25.

Thailand — Bangtrakulnonth A, Vieira AR, Lo Fo Wong DM a, Pornreongwong S, Pulsrikarn C, et al. (2008) *Shigella* from humans in Thailand during 1993 to 2006: spatial-time trends in species and serotype distribution. Foodborne Pathog Dis 5: 773–784.

The Gambia — Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, et al. (2014) *Shigella* isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis 59: 933–941.

Trinidad & Tobago — Orrett FA (2008) Prevalence of *Shigella* Serogroups and Their Antimicrobial Resistance Patterns in Southern Trinidad. J Health Popul Nutr 26: 456–462.

Tunisia — Al-Gallas N, Bahri O, Bouratbeen A, Haasen A Ben, Aissa R Ben (2007) Etiology of Acute Diarrhea in Children and Adults in Tunis,

	Tunisia, with Emphasis on Diarrheagenic Escherichia coli: Prevalence, Phenotyping, and Molecular Epidemiology. Am J Trop Med Hyg 77: 571–582.
Turkey	Saran B, Erdem B, Tekelİ FA, Şahİn F, Aysev AD (2013) Ankara' da İzole Edilen *Shigella* Kökenlerinin Antibiyotik Direnç Modelleri, Plazmid Profil Analizi ve Değişken Alanlı Jel Elektroforezi ile İncelenmesi (Characterization of *Shigella* Strains Isolated in Ankara, Turkey by Antimicrobial Resistance Models, Pl. Mikrobiyol Bul 47: 35–48.
United Arab Emirates	Jamal W, Rotimi VO, Pal T, Sonnevend A, Dimitrov TS (2010) Comparative in vitro activity of tigecycline and other antimicrobial agents against *Shigella* species from Kuwait and the United Arab of Emirates. J Infect Public Health 3: 35–42.
United Kingdom	Shigella cases: 1992 to 2013 (2013). Public Heal Engl Stat. Available: https://www.gov.uk/government/statistics/shigella-cases-1992-to-2013.
United States	National Antimicrobial Resistance Monitoring System: Enteric Bacteria (2011). Atlanta, Georgia. Available at http://www.cdc.gov/narms/pdf/2011-annual-report-narms-508c.pdf
Uruguay	Mota M, Gadea M, González S, González G, Pardo L, et al. (2010) Bacterial pathogens associated with bloody diarrhea in Uruguayan children. Rev Argent Microbiol 42: 114–117.
Uzbekistan	Madiyarov RS, Bektemirov AM, Ibadova G a, Abdukhalilova GK, Khodiev A V, et al. (2010) Antimicrobial resistance patterns and prevalence of class 1 and 2 integrons in *Shigella flexneri* and *Shigella sonnei* isolated in Uzbekistan. Gut Pathog 2: 18–24.
Venezuela	Pérez-Schael I, Salinas B, González R, Salas H, Ludert JE, et al. (2007) Rotavirus mortality confirmed by etiologic identification in Venezuelan children with diarrhea. Pediatr Infect Dis J 26: 393–397.
Vietnam	Vinh H, Nhu NTK, Nga TVT, Duy PT, Campbell JI, et al. (2009) A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. BMC Infect Dis 9.
Yemen	Al-Moyed K, Harmal N, Al-Harasy A, Al-Shamahy H (2006) Increasing single and multi-antibiotic resistance in *Shigella* species isolated from shigellosis patients in Sana'a, Yemen. Saudi Med J 27: 1157–1160.
Zimbabwe	Ndlovu N, Tarupiwa A, Mudzori J (2006) Antimicrobial resistance of *Shigella* species isolated during 2004 and 2005 from selected sites in Zimbabwe. Cent Afr J Med 52: 93–97.

Text S1. Methods for antimicrobial resistance and gene content data shown in Figure 2. A total of 136 *Shigella flexneri* and 231 *Shigella sonnei* isolates were collected between 1995 and 2010 as part of ongoing studies across Vietnam [1,2]. Antimicrobial susceptibilities were tested at the time of isolation by the modified Bauer-Kirby disk diffusion method, as recommended by the CLSI guidelines [3]. MICs were accessed by E-test, according to the manufacturer's recommendations (AB Biodisk). Mueller-Hinton agar and antimicrobial discs were purchased from Unipath, Basingstoke, United Kingdom. Escherichia coli ATCC 25922 was used as the control strain. The inhibitory zone sizes were recorded and interpreted according to current CLSI breakpoint guidelines [3]. The following antimicrobials were used for *Shigella*.spp. susceptibility testing: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), ceftriaxone (CRO), gatifloxacin (GAT), gentamicin (GEN), nalidixic acid (NAL), ofloxacin (OFX), trimethoprim/ sulfamethoxazole (SXT), tetracycline (TET) and trimethoprim (TMP).

Additionally, the isolates were subjected to DNA extraction by using Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA), and whole genome sequenced on an Illumina Hiseq2000 platform (Illumina, San Diego USA) to generate 150bp paired-end reads, as previously described [4]. *De novo* assemblies were generated for each read set using Velvet and VelvetOptimiser [5]. Contigs less than 100bp in size were excluded for further analysis. The short-read sequence data were deposited in the European Read Archive under the accession number ERP000182, ERP000631 and ERP000631. The resistance gene profiles and content (resistome) of each isolate were characterised using a manually curated database, based on the ResFinder database [6]. Each gene in the database was mapped against the isolate assemblies to identify complete genes. Where fragments matching the 5´or 3´ ends of resistance genes were identified at contig boundaries, sequencing reads were mapped to all matching candidate genes and their

presence assessed based on mapping coverage across the gene.

1. Holt K, Thieu Nga T, Thanh D, Vinh H, Kim D, et al. (2013) Tracking the establishment of local endemic populations of an emergent enteric pathogen. Proc Natl Acad Sci 110: 17522–17527.

2. Holt KE, Baker S, Weill F-X, Holmes EC, Kitchen A, et al. (2012) Shigella sonnei genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. Nat Genet 44: 1056–1059.

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7 RESEARCH PAPER 5: A cohort study to define the age-specific incidence and risk factors of *Shigella* diarrhoeal infections in Vietnamese children: a study protocol

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SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection and disease in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	BMC Public Health		
When was the work published?	2014		
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Where is the work intended to be published?	
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am first author. I worked with Stephen Baker on conceiving the design of the study, wrote the protocol and patient information materials, and oversaw the ethical approval process. I was heavily involved in study set up at the two participanting hospitals, including data and sample collection and management. I wrote the manuscript,

	generated all figures and was responsible for the submission process.

Student Signature: _____ **Date:** 16 Nov 2015

Supervisor Signature: _____ **Date:** 16/11/15

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BMC Public Health

STUDY PROTOCOL Open Access

A cohort study to define the age-specific incidence and risk factors of *Shigella* diarrhoeal infections in Vietnamese children: a study protocol

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Abstract

Background: *Shigella* spp. are one of the most common causes of paediatric dysentery globally, responsible for a substantial proportion of diarrhoeal disease morbidity and mortality, particularly in industrialising regions. Alarming levels of antimicrobial resistance are now reported in *S. flexneri* and *S. sonnei*, hampering treatment options. Little is known, however, about the burden of infection and disease due to *Shigella* spp. in the community.

Methods/Design: In order to estimate the incidence of this bacterial infection in the community in Ho Chi Minh City, Vietnam we have designed a longitudinal cohort to follow up approximately 700 children aged 12–60 months for two years with active and passive surveillance for diarrhoeal disease. Children will be seen at 6 month intervals for health checks where blood and stool samples will be collected. Families will also be contacted every two weeks for information on presence of diarrhoea in the child. Upon report of a diarrhoeal disease episode, study nurses will either travel to the family home to perform an evaluation or the family will attend a study hospital at a reduced cost, where a stool sample will also be collected. Case report forms collected at this time will detail information regarding disease history, risk factors and presence of disease in the household.
Outcomes will include (i) age-specific incidence of *Shigella* spp. and other agents of diarrhoeal disease in the community, (ii) risk factors for identified aetiologies, (iii) rates of seroconversion to a host of gastrointestinal pathogens in the first few years of life. Further work regarding the longitudinal immune response to a variety of *Shigella* antigens, host genetics and candidate vaccine/diagnostic proteins will also be conducted.

Discussion: This is the largest longitudinal cohort with active surveillance designed specifically to investigate *Shigella* infection and disease. The study is strengthened by the active surveillance component, which will likely capture a substantial proportion of episodes not normally identified through passive or hospital-based surveillance. It is hoped that information from this study will aid in the design and implementation of *Shigella* vaccine trials in the future.

Keywords: Diarrhoea, *Shigella*, Active surveillance, Vietnam, Cohort

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Background

Diarrhoea remains a major cause of childhood morbidity and mortality globally [1,2], with the vast majority of the 1.7 billion annual infections and 0.7 million deaths occurring in low and middle-income countries [3]. The four species of the Gram-negative bacterial genus *Shigella* (*S. flexneri, S. sonnei, S. boydii* and *S. dysenteriae*) are amongst the most common causes of dysenteric diarrhoea worldwide, with >164 million infections estimated annually in 1999 [4]. There is a growing necessity to prevent and control *Shigella* infections due to a dramatic emergence of highly resistant strains in not only southeast Asia [5-7], but in many other countries in both high and low income settings [8-16]. There are also extensive knowledge gaps regarding the distribution, incidence and exposure to *Shigella* spp., including a paucity of information on age-specific incidence, which is essential for assessing disease burden and evaluating the benefits of any future vaccines.

To address the outstanding epidemiological questions related to *Shigella* infections, we are conducting a longitudinal cohort study focusing on diarrhoeal disease with both routine and active surveillance components in Ho Chi Minh City (HCMC) in southern Vietnam. HCMC is a densely populated, rapidly urbanizing setting home to over 7.5 million people with a per-capita annual income of $1,220 [17]. The burden of *Shigella* infections is known to be substantial in children in Vietnam [5,18]. A previous cohort study conducted in the early 2000s in the coastal province of Khanh Hoa in central Vietnam estimated an incidence rate of 490/100,000/year in children under the age of five years through passive surveillance of diarrhoeal cases [19]. Although several longitudinal, community-based cohorts in Southeast Asia have been established within the last two decades to investigate diarrhoeal disease, very few have included an active disease surveillance component [19-24]. Active surveillance is challenging for obvious reasons, including the substantial cost, intensive staff and resource needs, difficulty in ascertainment and confusion on what is considered to be an 'episode' of diarrhoea by parents or guardians.

The overall aim of this study is to describe the epidemiology of *Shigella* infections in HCMC to inform the development and introduction of *Shigella* vaccines. The primary objective of this study will be to define the age-specific incidence and exposure of *Shigella* infection in children ≤ 60 months of age in the community in HCMC. Secondary objectives include (1) determination of the age-specific incidence of other agents of diarrhoeal infection in the community (2) a description of risk factors for both *Shigella* as well as other diarrhoeal aetiologies (3) evaluation of seroconversion rate (presence of antibody) to diarrhoeal pathogens at six month intervals in the first five years of life (4) exploration of the relationship between maternal and infant IgG and protection against infection and (5) an investigation into potential host genetic susceptibilities for diarrhoeal disease.

Methods/Design

In order to achieve our objectives, we have designed a longitudinal cohort study with routine and active surveillance for diarrhoeal disease in district eight of HCHC (Figure 1). This district is a densely populated district in the center of the city with a large number of canals and waterways and is known to contribute proportionally more hospitalized pediatric shigellosis patients than any of the other districts in the city [5].

Cohort population

Children enrolled into our cohort will be recruited from an existing birth cohort conducted to investigate the incidence of dengue and other viral infections across our study site [24]. Briefly, pregnant mothers living in district eight were enrolled prior to delivery at Hung Vuong Hospital (HVH), a large obstetric hospital in HCMC delivering approximately 44,000 babies annually. Infants were followed up routinely for the first 12 months of life for the purposes of investigating the epidemiology, aetiology and risk factors of a variety of viral infections in infancy. Clinical samples were collected at routine visits corresponding to Expanded Programme on Immunization (EPI) visits at 0, 4, 9 and 12 months of age, including throat and nasopharyngeal swabs as well as a small blood sample. A subset of these children have been followed beyond 12 months of age and are still undergoing routine follow up every six months.

Enrolment & routine follow up

Children who had been enrolled in the existing birth cohort will be approached for entry into the diarrhoeal disease cohort between 12–36 months of age at a routine birth cohort follow up visit. An enrolment questionnaire will be administered, detailing information on demographic and socioeconomic characteristics of the household, with a particular focus on water sources, water treatment and toilet practices. Once enrolled, children are requested to attend HVH every six months for a routine health check, as shown in Figure 2. A blood sample (<2 ml), a nasopharyngeal swab and an anal swab or stool sample (if available) is collected by study nurses at these routine visits. Additionally, a questionnaire regarding growth and disease episodes of the child in the preceding six months will be administered. Routine follow up will take place for a total of two years for each child.

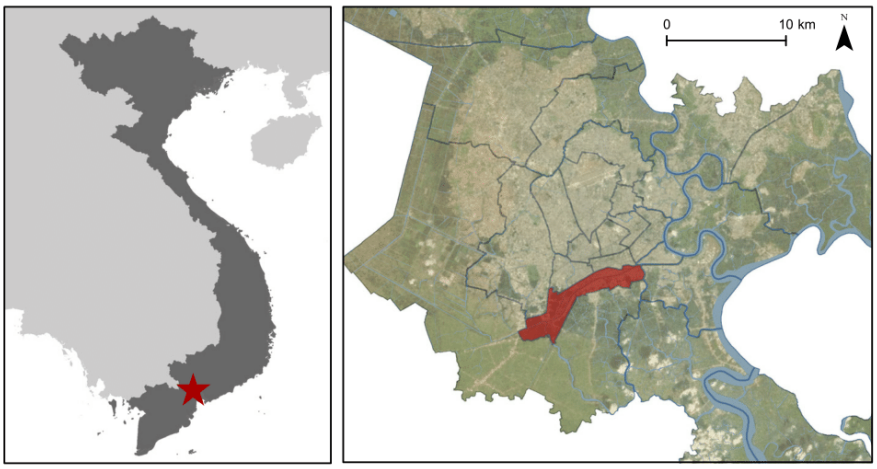

Figure 1 The location for the cohort study. Left panel: Location of Vietnam (dark grey) in Southeast Asia, with Ho Chi Minh City shown by the red star. Right panel: A satellite map showing Ho Chi Minh City, with district 8 highlighted in red. The boundaries of other districts are shown in black. Waterways are shown in blue.

Active & passive case detection

Parents/guardians will be contacted through a text message or phone call on a regular basis to request information on whether the enrolled child has had an episode of diarrhoea in the preceding seven days. Diarrhoea is defined according to the guidelines outlined by the World Health Organization, which is three or more loose stools in a 24 hour period or at least one bloody/mucoid stool [25]. If the parent cannot be reached on the initial attempt, the study nurse will make every effort to establish contact to ensure complete data collection. Once a parent alerts a study nurse to an episode of diarrhoea, the nurse will travel to the participant's home within a 24-hour window. At the home visit, a stool sample will be collected. If no fresh stool is available at the time of the visit, a sterile stool collection pot will be left with the parents/guardians with instructions for sample collection. Stool pots left at the home will be collected within 24 hours. Furthermore, a short case report form (CRF) will be collected detailing clinical details of the diarrhoeal episode and information on any other known diarrhoeal infections in the household. The parents will also be given oral rehydration packets, probiotics containing *Lactobacillus acidophilus* as well as zinc supplements for the child [26,27].

If at any point during follow up a parent feels that their child requires medical attention for diarrhoeal disease and feels that a home visit by a nurse is insufficient, they may attend the Hospital for Tropical Disease (HTD) at a subsidised cost (Figure 2). Upon arrival at the hospital the children will be escorted through registration processes by a study nurse who will then lead them to the gastrointestinal ward for clinical evaluation (both inpatient and outpatients). A stool sample will be collected, and a

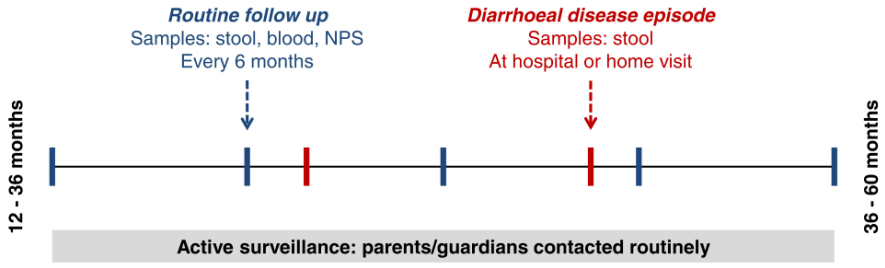

Figure 2 Flowchart of study design. Children are enroled at age 12–36 months with routine follow up visits (shown in blue bars) every 6 months. During enrolment and routine follow up visits, a stool sample, blood sample and nasopharyngeal swab (NPS) will be collected in addition to a case report form detailing growth and health information. Follow up will continue for a total of two years for each child, meaning they will exit the cohort at 36–60 months of age. Parents will be contacted on a regular basis to enquire whether their child has experience diarrhoeal disease. When the child experiences an episode of diarrhoea (shown in red bars), study nurses can travel to the home of the participant to a stool sample and a short case report form or the family can bring the child to the Hospital for Tropical Disease for clinical examination. Stool will be collected during diarrhoeal episodes.

blood sample if clinically indicated. A detailed CRF will be administered collecting clinical, laboratory and haematological information if available.

Clinical and laboratory investigations

All samples will be labeled with a study number to ensure anonymity and transported to the microbiology laboratory at the Oxford University Clinical Research Unit (OUCRU) for analysis and storage on the same day as collection. Samples from disease episodes will undergo microscopy for blood cells and parasites (*Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium*) as well as microbiology culture for *Shigella* spp., *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli*. Antimicrobial susceptibility testing of the cultured and identified bacterial pathogens will be performed by the disc diffusion method using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [28]. Results will be reported back to the treating clinician as soon as they are available.

Aliquots of stool from both healthy routine visits and from disease episodes will be stored at −80°C. Total nucleic acid will be extracted from faecal specimens using the QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) or using the Roche MagNA pure 96 automated nucleic acid extraction machine (Roche). RNA will be converted to complementary DNA (cDNA) by reverse transcription (RT) and an aliquot of RNA will be stored −80°C. For RT, extracted RNA will be reverse-transcribed by SuperScript Reverse Transcriptase III and RNAse Inhibitor (Invitrogen) combined with a random hexamer (Roche Diagnostics, UK) according to manufacturer's instructions. The resulting cDNA will be stored at −80°C. Bacterial PCR will be performed on extracted nucleic acids to potentially increase the diagnosis rate of *Shigella* spp., *Salmonella* spp. and *Campylobacter* spp. Viral pathogens will be identified through batch multiplex realtime PCR procedures to identify rotavirus and norovirus [29]. Additionally, blood samples will be separated into plasma for serology purposes and cells will be stored for future host genetics studies. Specifically, ELISAs will be used to measure IgA, IgM and IgG subtypes to *S. sonnei* O-antigen to evaluate seroconversion rates [30].

Sample size

We estimate that we will enroll between 650–750 children based on the available population and current attrition rates from the original birth cohort [24]. It is known that the median age of hospitalized *Shigella* infections in HCMC is 24 months [5]. Additionally, the annual incidence of diarrhoea in Vietnam in children under five years of age is 1.5 events/child/year [19]. Therefore, we estimate that we will have 2,100 diarrhoeal episodes in our cohort during two years of follow up. From a cohort study in Hanoi, the *Shigella* positivity rate is approximately 6% of passively detected diarrhoeal cases in children under 5 years [20]. Therefore, we conservatively estimate that we will detect approximately 125 *Shigella* diarrheal episodes over the study period through both passive and active surveillance activities.

Data management

Each patient will have a unique identifying study code such that sample and documents will not be labeled with any identifying information. Data will be collected electronically whenever possible, including on laptops and on handheld tablets provided to the study nurses and hospital wards. Electronic data entry devices will be password protected and accessible only by authorised users. Paper CRFs will be used in the event of power failure or technical difficulty. A central database has been developed to ensure secure and confidential data management.

Ethical approval & ethical considerations

Ethical approval for this study has been obtained from the Oxford University Tropical Research Ethics Committee (OxTREC approval 1058–13) as well as from local partners including the Institutional Review Board (IRB) at the Hospital for Tropical Diseases (HTD) and the IRB at Hung Vuong Hospital (HVH). Written informed consent will be obtained from parents/guardians of children at the time of enrolment for both participation as well as storage and future use of pathogenic and human samples. Parents/guardians will be assured that all information generated in this study will remain confidential. Participant families are reimbursed for travel costs to our study sites for routine or disease visits as well as tests involved in the standard of care (full blood count, stool microscopy and microbiology) of diarrhoeal disease at HTD. Furthermore, parents/guardians are free to withdraw their consent for their child at any time and request that study samples not be stored for further testing.

Discussion

In this work we have described a longitudinal cohort designed to estimate the incidence of diarrhoea due to *Shigella* in the community in HCMC, Vietnam. This study will follow an estimated 650–750 children under the age of five for two years each, collecting information at routine follow up visits and during diarrhoeal disease events through both passive and active surveillance. We aim not only to estimate the incidence of *Shigella* but also to examine the immune response and risk factors for infection and disease.

Study strengths

The burden of diarrhoeal disease is difficult to accurately estimate [31], and hospitalized illness is generally not representative of infection and disease in the community [32]. Therefore, the most important strength of the study is the active surveillance component as we will be able to estimate the true burden of diarrhoea due not only to *Shigella*, but due to a variety of other diarrheal aetiologies in the community as well. Longitudinal blood samples will also provide for the ability to estimate rates seroconversion to *Shigella sonnei* O-antigen [33], which will capture potential *Shigella* infections that we do not identify through our active and passive surveillance. An additional strength of this study is the collaboration with the existing birth cohort established at OUCRU [24]. Such a partnership will allow for data and sample sharing for all participants from birth through at least the first three years of life, providing for the opportunity to explore dynamics of infection and immunity from infancy.

Limitations

The major, predicted limitations of this study are loss to follow up and incomplete diarrhoeal disease ascertainment. Differential loss to follow up would introduce bias into the results as participants who remain are likely different than those who were lost. Therefore, we will ensure that the rapport between study nurses and participants remains strong through continuous training and maintenance of motivation of the nurses. We have made a significant effort to provide the families with a high level of comprehensive care for diarrhoeal disease in hopes that they will value the overall research programme and continue to participate through the follow up period. Additionally, we are investing heavily in terms of time and personnel to ensure that we capture as many diarrhoeal disease episodes as possible through intensive active surveillance and follow up.

Future work

The humoral immune response to *Shigella* is partly induced by the O-antigen and is thought to be serotype specific [34,35]. However, the immune response to the O-antigen as well as other non-polysaccharide *Shigella* antigens in children in endemic areas after symptomatic infection or exposure is extremely ill-defined [36]. We will explore the longitudinal immune response to a range of *Shigella* antigens in infected individuals in an endemic setting with a view of identifying novel vaccine and diagnostic candidates and their serological relationship to the O-antigen. To interrogate the diversity, specificity and longevity of the humoral immune response a range of *Shigella* antigens, a *Shigella* antigen-array will be constructed in collaboration with the Wellcome Trust Sanger Institute. Similar approaches have been used for development of other bacterial vaccines and diagnostics such as *Salmonella* and *Brucella melitensis* [37,38].

Additionally, we hope to explore the relationship between genetic variation and disease susceptibility through genetic association, candidate gene and genome wide association studies in the future. Host DNA samples will be stored and used for genotyping to investigate potential genetic associations with diarrhoeal disease. Additionally, the development of gastrointestinal and respiratory tract flora will be measured by performing metagenomic analysis from the anal and nasopharyngeal swabs.

Conclusions

In conclusion, we have designed one of the largest longitudinal, active surveillance cohort studies to study *Shigella* infection and disease in children in Southeast Asia. Through this study, we will be able to estimate an incidence of *Shigella* infection and disease and define the epidemiology of the bacteria in this community, which is representative of many densely crowded, industrializing cities globally. We also hope to eventually more clearly define the longitudinal immune response to this emergent, highly antimicrobial resistant pathogen [12], as well as begin exploration into host susceptibility to infection. Samples, data and analyses from this work are hoped to be a valuable resource to international as well as local medical and public health communities for informing the development and deployment of a *Shigella* vaccine in the future.

Abbreviations

CLSI: Clinical laboratory standards institute; CRF: Case report form; EPI: Extended programme on immunization; HCMC: Ho Chi Minh City; HTD: Hospital for tropical disease; HVH: Hung Vuong Hospital; NPS: Nasopharyngeal swab; OUCRU: Oxford university clinical research unit; RT: Reverse-transcribed.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Designed the study: CNT, KLA, CS, SB, NVVC, GT. Microbiology and laboratory support: HTT, PVM, LTPT, TDHN, JIP. Coordination of study at OUCRU and study sites: LTQN, VTD, LLV, NTVT, HNT. Study nurse: NTTN, TTTL. Wrote the manuscript: CNT, SB. All authors read and approved the final manuscript.

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8 RESEARCH PAPER 6: Active surveillance for diarrhoeal disease in the community: incidence of *Shigella* and other enteric infections in Ho Chi Minh City

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SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection and disease in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	The Pediatric Infectious Disease Journal
Please list the paper's authors in the intended authorship order:	Corinne N. Thompson, Le Thi Quynh Nhi, Katherine L. Anders, Nguyen Thi Thanh Nhan, Tran Thi Thao Ly, Nguyen Trong Hieu, Lu Lan Vi, Nguyen Van Vinh Chau, Vu Thuy Duong, Le Thi Phuong Tu, Nguyen Ngoc Minh Chau, Ha Thanh Tuyen, James Campbell, Pham Van Minh, Tran Do Hoang Nhu, Guy Thwaites, Cameron P. Simmons and Stephen Baker
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am first author. I wrote the first draft of the protocol, was heavily inovlved in ethical approval processes, led the daily management of the cohort study and oversaw data and sample management. I was responsible for database cleaning and development of analysis plans. I wrote the STATA code for all analyses and the R code for all figures. I wrote the manuscript.

Student Signature: _____ **Date:** 16 NOV 2015

Supervisor Signature: _____ **Date:** 16/11/15

Title: Active surveillance for diarrhoeal disease in the community: incidence of *Shigella* and other infections in Ho Chi Minh City

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Running title: *Shigella* diarrhoeal cohort in Vietnam

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Abstract

Background: Diarrhoeal disease remains a significant cause of childhood morbidity and mortality globally. *Shigella* spp. are a genus of Gram-negative bacteria that cause bloody/mucoid diarrhoea with potentially severe consequences in young children. Antimicrobial resistance is a growing threat, and little is known about *Shigella* or other agents of diarrhoeal disease in the community.

Methods: Children between the ages of 12-36 months were enrolled into a large community cohort with active surveillance for diarrhoeal disease in Ho Chi Minh City, Vietnam. Clinical samples were collected at routine follow up visits and during diarrhoeal disease episodes. A multiplex molecular diagnostic platform was used to identify >15 pathogens with high sensitivity and specificity.

Results: For the first year of the cohort, a total of 748 children were enrolled and the incidence of diarrhoeal disease was 70 episodes/100 child years of observation (CYO). Norovirus was most common (13 episodes/100CYO) followed by *Salmonella* (9 episodes/100CYO). The incidence of *Shigella* was 1.5/100CYO and was similar in both two and three year olds. Malnourished children in the cohort were significantly more at risk of developing a *Shigella* infection.

Conclusions: Diarrhoeal disease in the community in Ho Chi Minh City is most frequently caused by norovirus in both two and three year olds. *Shigella* infections still plague the malnourished. The community cohort structure of this study will permit a wide range of future analyses ranging from immunology to host genetics to better understand *Shigella* and diarrhoeal disease as a whole.

Introduction

Diarrhoea remains a substantial cause of morbidity and mortality amongst children globally [1,2]. *Shigella*, a major cause of bacillary dysentery, is a considerable public health problem in many regions, particularly in the second and third year of life [2,3]. Infections in young children can result in severe acute illness as well as significant reductions in linear growth during childhood [3–5]. Of the four species of *Shigella*, *S. sonnei* is emerging in many regions traditionally dominated by *S. flexneri* that are undergoing economic industrialisation [6]. Reasons behind this emergence are unclear [7], yet due to alarming levels of antimicrobial resistance against first and second line therapies [8–11], *S. sonnei* is now a formidable threat in areas such as Asia, Latin America and the Middle East [12–17]. Although vaccines are under development, a safe and effective candidate has yet to be licensed [18].

Vietnam is a lower middle income country with a rapidly industrialising population of 90 million people and dual burdens of both infectious and non-communicable diseases [19]. Ho Chi Minh City (HCMC), located in the tropical south, is the largest city in Vietnam and has a considerable burden of diarrhoeal disease [20–22]. Although paediatric *Shigella*, rotavirus and norovirus patients are known present commonly to hospitals in this setting [21], little is known about the true burden of disease in the community. A study conducted in 2001-2003 in the central, coastal province of Khanh Hoa in Vietnam found the incidence of diarrhoea and *Shigella* in children aged under five years to be 11.5/100 and 4.9/1000 per year, respectively [23].

More recently, a birth cohort in southern Vietnam quantified the incidence of diarrhoeal disease in infants <12 months of age to be 271/1000 infant years of observation (IYO), with significant differences between HCMC (89/1000 IYO) and the rural Dong Thap province (604/1000 IYO), located in the Mekong Delta [24]. Both of these studies relied on passive, clinic-based diarrhoeal disease detection, so likely represent underestimates of the true burden of both diarrhoea generally and *Shigella* specifically. Therefore, we conducted a longitudinal, community-based cohort using

active surveillance for diarrhoeal disease to more accurately estimate the burden of *Shigella* and other diarrhoeal infections in this setting [25].

Due to known high levels of antimicrobial usage in the community in Vietnam [26,27], we detected an array of diarrhoeal pathogens using both traditional microbiological methodology in addition to molecular techniques to increase our sensitivity of detection during a diarrhoeal episode. Molecular diagnostic technology such as the Luminex xTAG Gastrointestinal Pathogen Panel system dramatically expands the ability to diagnose a large variety of viral, bacterial and parasitic infections in a period of hours [28]. Luminex relies on molecular amplification of specific regions of the genome of a variety of pathogens, which are then hybridised to pathogen-specific beads for detection and quantification by fluorescence. With the ability to identify up to 21 different enteric pathogens and with sensitivity and specificity exceeding 90% for a majority of targets in the Vietnamese setting (Duong et al, manuscript in preparation), this system has been also shown to be effective in a variety of high-income settings [29–31] and provides an unprecedented level of diagnostic scope and sensitivity.

The overall aim of this study was to describe the epidemiology of *Shigella* infections in HCMC to inform the development and potential introduction of *Shigella* vaccines. We report age-specific incidence of *Shigella* and other diarrhoeal pathogens using molecular diagnostics from the first year of a cohort study employing active surveillance for diarrhoeal disease in the community. Furthermore, we examine clinical manifestations, rates of coinfection, seasonality and risk factors of various pathogens to develop a better understanding of diarrhoeal disease occurring in the community an industrialising country in Southeast Asia.

Methods

Cohort design

The protocol for this diarrhoea cohort has been published previously [25]. Briefly, 748 children between the ages of 12-36 months were enrolled in a community cohort based in district 8 of HCMC. Children were identified for screening if they had participated in a previous birth cohort

[32], which enrolled pregnant mothers at Hung Vuong Hospital who lived in district 8 of HCMC. In this original birth cohort, children were followed up for one year with regular health checkups and passive disease surveillance [24]. When children finished the original birth cohort at 12 months of age they were eligible for enrolment into the diarrhoea cohort with active disease surveillance. Once enrolled, these children were followed up for two years with routine health checkups every six months. At routine follow up visits, a rectal swab, nasopharyngeal swab and small blood sample were collected, along with information regarding growth metrics and any unrecorded hospitalisations in the interim period. For the active surveillance component, an SMS was sent to each participant biweekly enquiring as to whether the study child had experienced diarrhoea in the last seven days. For those who did not reply, phone calls were made to established presence of diarrhoea in the preceding seven days. Diarrhoea was defined as three or more loose stools in a period of 24 hours or one stool of bloody or mucoid diarrhoea (dysentery) [33]. If diarrhoea was reported, a study nurse went to the participant's home or the family was invited to attend the Hospital for Tropical Disease for examination. When ill, a stool sample was collected and a clinical case report form administered.

Clinical specimen collection and traditional microbiology

Stool samples were collected in a sterile pot and transported within 24 hours of collection. Classical microbiological culturing was performed on all collected fresh stool samples on the day of sampling to isolate common diarrheal bacteria including *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Plesiomonas* spp. and *Aeromonas* spp. as described previously [34]. Specific serotypes of *Shigella* spp. and *Salmonella* spp. were identified by slide agglutination with antigen grouping sera and monovalent antisera, and *Campylobacter jejuni* was differentiated from *Campylobacter coli* by the hippurate hydrolysis test as previously described [34]. A fresh smear of faecal specimen was prepared in phosphate buffered saline to examine the presence of *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium* cysts [34].

Nucleic acid extraction, multiplex PCR and Luminex procedures

A total of 200µl of each stool specimen was used for nucleic acid extraction with 10µl xTAG MS2 (internal control) by MagNA Pure-96 machine (Roche) according to the manufacturer's instruction. Each 15µl-master mixture contained 7.5µl of 5X xTAG One Step Buffer, 2.5µl of xTAG GPP Primer Mix, 0.5µl of xTAG BSA (10mg/ml), 2µl of xTAG One Step Enzyme Mix, and water up to 15µl. A total of 10µl of the appropriate extracted nucleic acid sample was used as template for the PCR step. The PCR reaction was performed at 53°C for 20 minutes and the thermal cycler conditions used were enzyme activated at 95°C for 15 min; 95°C for 30s; 38 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s; a final extension at 72°C for 2 min; and holding at 4°C.

The xTAG GPP Bead mix and Reporter Buffer containing 0.15M NaCl were thawed and the 0.22 SAPE was diluted 75-fold with the xTAG Reporter Buffer and kept away from light until use. Each reaction contained 20µl xTAG Bead Mix, 5µl PCR product and 75µl of reporter solution. The hybridisation was performed at 60^0C for 3 min, 45^0C for 45 min, and holding at 45^0C. When the hybridisation was complete, the plate was placed on the Luminex plate heater block and read by Luminex® 100/200™ software.

Statistical analyses

A new episode of diarrhoea was defined by ≥7 days between the onset dates of symptoms. Incidence was calculated by dividing the number of distinct episodes by individual child follow up time and reported as a population mean with 95% confidence intervals (CIs). Univariate logistic regression was performed to examine pathogen-specific risk factors. Cases included any individual who had at least one recorded pathogen-specific diarrhoeal episodes diagnosed by the Luminex assay and controls included the remaining children in the cohort, whether diarrhoea-positive for another pathogen or diarrhoea-negative throughout the study period. Longitudinal prevalence was estimated by using the number of positive SMS/calling responses divided by the total number of weeks under observation. As the SMS asked explicitly about diarrhoea in the last 7 days, one SMS equated to one week under observation. Analyses were performed in STATA

v13.1 (College Station, TX, USA) and plots were generated in R (R Statistical Foundation for Computing, Vienna, Austria; https://cran.r-project.org/) using the *ggplot2* package [35].

Ethical approval

The Hospital for Tropical Diseases and Hung Vuong obstetric hospital participated in the study. The protocol was approved by the institutional review boards of these hospitals as well as the Oxford Tropical Research Ethics Committee (OxTREC approval: 0209) and the London School of Hygiene & Tropical Medicine (Ref: 8632). Written informed consent was obtained from all participants.

Results

Baseline

From June 2014 – July 2015 there were 748 children aged 12-36 months who were enrolled into the diarrhoea cohort. The median age of children at enrolment was 24 months (interquartile range [IQR]: 13-30 months), with 53% (398/748) male as shown in Table 1. At least 42% (314) of children reported at least one dose of a rotavirus vaccine, the first dose of which was at a median of two months of age (IQR: 2-3)**.** The median time of follow up in the cohort in the first year was 271 days (range: 137-365) and 15 children (2%) dropped out or were lost to follow up. The most common reason for dropping out was dislike of blood collection (10/15, 67%). There were no major socioeconomic differences between those that dropped out (2%) and those that remained in the cohort within the first year of enrolment and follow up (data not shown). A total of 244 (33%) children had at least one home or hospital visit or self-reported hospitalisation for diarrhoea and 149 (20%) had at least one stool sample collected during a diarrhoeal episode throughout follow up.

Incidence and longitudinal prevalence of diarrhoeal episodes

During the first 12 months of the cohort study, there were 400 reported diarrhoeal episodes: 227 (57%) were home visits, 166 (42%) were hospital visits at the HTD and 6 (2%) were self-reported

hospitalisations for diarrhoea at a hospital other than HTD. Stool was more often collected at HTD hospital visits (163/166, 98%) compared to during home visits (71/227, 31%) as many home visits were recorded only after the seven day window of a dirrhoeal episode. Only 12 (4.8%) episodes were home visits that went on to be hospitalised. There were a total of 558.9 child years of follow up recorded, leading to an overall incidence of 69.6 (95%CI: 59.2-80.0) diarrhoeal episodes per 100 child years of observation (CYO). Diarrhoeal episodes treated at home had an incidence of 40.5 (95%CI: 34.4-46.5) per 100 CYO and those treated in hospital 27.6 (95%CI: 20.1-35.1) per 100 CYO.

Over the 12 months of follow up, there were 12,636 SMS sent biweekly to participants (median 18 SMS/participant, range: 4 – 23), with an SMS response rate of 35%. From the SMS and calling, there were 292 diarrhoeal episodes reported. The longitudinal period prevalence of diarrhoeal disease is therefore estimated to be 2.7% (95%CI: 2.2-3.1%), indicating that a child in this cohort will report diarrhoea on 1.4 (95%CI: 1.2-1.6) weeks in a one year period.

Pathogen and age-specific incidence

Using the Luminex platform, a variety of pathogens were identified in diarrhoeal stool samples as shown in Table 2. Diarrhoeal disease due to norovirus was most frequent (12.5 episodes [95%CI: 8.4-16.5] per 100 CYO), followed by *Salmonella* spp. (8.8 episodes [95%CI: 5.8-11.9] per 100 CYO) and *C. difficile* (7.4 episodes [95%CI: 4.8-9.9] per 100 CYO). The incidence of *Shigella* spp. as 1.5 episodes (95%CI: 0.5-2.6) per 100 CYO. The burden of disease detected through traditional microbiological culture methods was markedly reduced compared to the Luminex platform (Table 2). By age, children 12-23 months had the highest incidence of diarrhoeal disease generally (80.8 episodes [95%CI: 63.9-97.7] per 100 CYO) and similar burdens of both hospitalised (38.5 episodes [95%CI: 25.6 – 51.3] per 100 CYO) and home-treated (40.8 episodes [95%CI: 32.2-49.3] per 100 CYO) diarrhoeal disease. However, by the time children were ≥36 months, home-treated diarrhoeal disease (19.3 episodes [95%CI: 8-30.5] per 100 CYO) were

much more frequent than hospitalised diarrhoeal disease (8.8 episodes [95%CI: 2.5-15.1] per 100 CYO).

The distribution of pathogens across age groups was interesting for several reasons (Table 3, Figure 1). First, norovirus was the most commonly identified aetiology in both two and three year old children, found in 22% (54/258) and 18% (22/121) of diarrhoeal stool samples, respectively. Secondly, *C. difficile* had an incidence of 12 episodes (95%CI: 7.6-16.5) per 100 CYO in two year olds, yet was rarely identified in older children. While the incidence of most pathogens declined between the second and third years of life, the incidence of *Shigella*, though low overall, was similar in both two (1.5 episodes per 100 CYO) and three year olds (2.1 episodes per 100 CYO).

Coinfection

Of all 304 stool samples, a pathogen was detected in 223 (73%). A coinfection (>1 pathogen) was identified in 41% of stool samples with a detected pathogen (91/223). Coinfections were more common in diarrhoeal samples collected during home-treated diarrhoea (32/54, 59%) compared to hospitalised cases (52/129, 43%) (p=0.042, chi square test). The most common coinfection phenotype was norovirus/*Salmonella* (11%; 10/91) followed by norovirus/*C. difficile* (7%; 6/91) and norovirus/ETEC (7%; 6/91). As shown in Figure 2, *Cryptosporidium* (11 coinfections/11 positive samples, 100%), ETEC (26/32, 81%), *Salmonella* spp. (41/56, 72%) and adenovirus (25/35, 71%) were commonly isolated in coinfections. Rotavirus (12 coinfection/32 positive samples, 38%), *Shigella* (4/10, 40%), *Campylobacter* (12/27, 44%) were least likely to be detected amongst a coinfection. Rates of coinfection were not different between age groups (data not shown).

Reinfection

Multiple episodes of the same pathogen in a single participant were most frequent with norovirus (8/56 patients, 14%), adenovirus (3/25 patients, 12%), *C. difficile* (4/36 patients, 11%) and *Salmonella* spp. (4/44 patients, 9%) with median gaps between episodes of 19 days (IQR: 10-63),

39 days (IQR: 11-51), 66 days (51-122) and 51 days (34-88), respectively. The median number of episodes per patient for these four pathogens was 2-2.5, with a range of 2-7 episodes/patient for both norovirus and *Salmonella* spp., 2-4 episodes/patient for adenovirus and 2-3 episodes/patient for *C. difficile.* None of the patients infected with *Shigella* had multiple episodes (0/9).

Seasonality

As shown in Figure 3, several pathogens displayed an element of seasonality. Norovirus, for example, had a very sharp peak in incidence from August-October 2014, ranging from 23-40 episodes/100 CYO. The overall count of norovirus cases, however, remained consistently high from August 2014 – March 2015, with a median episode count of 8.5 (IQR: 7-9.3) per month. *Salmonella* episodes also displayed strong seasonality in early autumn 2014, with incidence peaking at 44 episodes/100 CYO in August 2014. Finally, the incidence of *C. difficile* peaked August –November 2014, ranging from 12-25 episodes/100 CYO with a median episode count of 6 episodes (IQR: 5.3-7.3) per month.

Clinical manifestations

Overall, 12% (20/165) of hospitalised and 0.4% (1/227) of home-treated diarrhoeal episodes had evidence of blood in the stool. A further 61% (100/165) of hospitalised and 17% (38/227) of home-treated episodes had mucoid diarrhoea. Over half (59%; 97/167) of diarrhoeal episodes treated in hospital were prescribed an antimicrobial. Macrolides were most commonly prescribed in hospital (34/87, 39%), followed by fluoroquinolones (24/87, 28%). For diarrhoeal illness at home, parents reported self-prescribed antimicrobial use in 25% of episodes (56/226) and probiotic use in 53% (119/226). Furthermore, 7% (16/227) of diarrhoeal episodes seen at home had a concomitant case of diarrhoea in the household, with the median age of the additional cases 23 years (IQR: 2-44). As shown in Table 4, *Shigella* monoinfections commonly presented with mucus or blood in the stool, abdominal pain and had the highest median axillary temperature on entry (38.5°C) compared to other diagnosed aetiologies. Additionally, viral infections tended to present with vomiting more frequently than bacterial infections.

Risks for pathogen-specific infections

Basic univariate analyses demonstrated several risk factors for pathogen-specific diarrhoea. As shown in Tables 1 and 5, low weight and height for age Z-scores at 12 months of age were significant risks for *Shigella* infection. Younger children were significantly more likely to present with *C. difficile, Salmonella* spp. and norovirus. Male sex was also a significant risk for *C. difficile,* norovirus and rotavirus infections. Additionally, using the floor for defecation instead of a flush toilet was a significant risk for *Salmonella* spp. and adenovirus infections, whilst wearing a diaper was associated with *Salmonella* spp. and norovirus infections after controlling for age. Finally, regular consumption of probiotics was a significant risk factor for rotavirus infections.

Discussion

Diarrhoeal disease continues to persist as a significant cause of childhood morbidity globally [36]. Through a longitudinal community cohort in HCMC we have quantified the incidence of diarrhoea disease in children between 12-48 months to be 70 episodes/100 CYO and document a range of aetiologies present in the stool of ill children. Our overall incidence estimate is much higher than that found in the coastal city of Nha Trang (11.5/100 CYO) in children under 5 in the mid-2000's [23]. This is likely due to differing strategies for diarrhoeal episode capture. Indeed, the rate of home-treated diarrhoeal episodes was almost 1.5 times as great as the rate of episodes treated at hospital, demonstrating that accurately quantifying diarrhoeal incidence in the community requires active surveillance. Yet our estimate is lower than that of Isenbarger *et al,* who found an incidence in Hanoi of 1.3 episodes/child/year in the late 1990s in children under five years of age [37], which likely reflects a true trend in declining morbidity due to diarrhoeal diseases in Vietnam over time as the country industrialises.

Although the incidence of *Shigella* (1.5 episodes/100 CYO) was low in comparison other diarrhoeal aetiologies identified in the current cohort study, our *Shigella* incidence estimate is three times of that of a large study conducted a decade ago in central Vietnam and is in fact most similar to that of Indonesia (1.9/100/year), Pakistan (1.7/100/year) and China (1.9/100/year) from

the same study [23]. As the median age of children currently in the cohort was 24 months, half of our cohort has yet to progress through the age where *Shigella* infections are known to be more common (2-3 years) [2,3,21], suggesting that our incidence estimate will likely grow as the cohort matures. However, even with the limited number of *Shigella* episodes identified in the first year, children with *Shigella* were significantly more likely to be stunted at 12 months of age and presented very frequently with dysentery (blood and/or mucus in stool), demonstrating the relative severity and continuing burden of this bacterial infection in malnourished children [38].

The use of the Luminex platform allows for a high level of diagnostic granularity with high sensitivity and specificity in a period of hours [29,39,40]. Through this assay we identified a large range of pathogens in the stool of children with diarrhoea, including most commonly norovirus, *C. difficile*, rotavirus, adenovirus and ETEC in the second year of life and norovirus, adenovirus, ETEC, *Campylobacter* spp. and *Shigella* spp. in the third year of life, similar to the GEMS and MAL-ED studies [2,3]. Yet, unlike the GEMS, we did not identify a particularly high incidence of *Cryptosporidium* (1.7 episodes/100 CYO), though this may be due to limited follow up time in our cohort. Furthermore, the ability to identify the aetiologies present in coinfection in addition to future work examining healthy stools of our cohort children will allow us to investigate the relative pathogenicity of each aetiology. In previous hospital-based studies in HCMC we identified *Salmonella* and *Campylobacter* spp. in the stools of asymptomatic controls under five years of age [21].

Though the number of diagnosed diarrhoeal episodes was relatively limited, we were still able to identify several risk factors with tangible preventative solutions, such as floor defecation in the instance of *Salmonella* and adenovirus, or the use of diapers again in *Salmonella* and norovirus infections. Encouraging the use of chamber pots for small children and likely proper hand hygiene when handling soiled diapers may prevent transmission of such pathogens within the home. More thorough analyses are warranted. Additionally, we found that rotavirus vaccination did not effectively prevent rotavirus disease in these cohort children and furthermore that probiotic consumption on a regular basis was actually a significant risk for rotavirus diarrhoea. Whether

such an association represents an interaction between probiotics and gut microbiota that would render a child more susceptible to rotavirus infection merits further study.

One of the limitations of the present study was the frequency of follow up. We chose biweekly follow up as a compromise between impacting the behaviour of the participants, time availability of the study nurses and annoying the participants to the point where they drop out [41]. Furthermore, it has been shown that frequently surveying for diarrhoeal disease actually leads to reduced incidence of reporting [42]. Even with biweekly surveillance, we found a drop in the overall reporting of diarrhoea over time which may have led to underestimated rates of diarrhoeal disease. While our loss to follow up rate was low (2%), we acknowledge that we were unlikely to obtain information on all episodes of diarrhoea that occurred in our cohort. While our active surveillance strategy was able to capture substantially more episodes than passive surveillance [24], it will be important to evaluate the seroepidemiology of our cohort members to investigate seroconversion rates [43], which may provide a more accurate burden estimate.

The rich dataset of this cohort study will permit a number of future analyses, including evaluation of the relative pathogenicity of the variety of identified aetiologies through examination of healthy stool samples as was done recently in the MAL-ED study [3]. We also aim to investigate the longitudinal immune response to a variety of aetiologies in addition to the development of an antigen array designed to identify immunogenic proteins for *Shigella* spp. [44]. Furthermore, we will perform a more rigorous analysis of risk factors, including a geospatial analysis [45], upon the completion of the cohort. Finally we will use clinical samples collected through this cohort to explore the relationship between genetic variation and disease susceptibility through genetic association, candidate gene and genome wide-association studies in the future [25].

In conclusion, through the structure of a large, longitudinal community-based cohort study we show the incidence of diarrhoeal disease in children between 12-48 months of age to be 70 episodes/100 CYO and highlight the diversity of pathogens identified in stool of sick children. Norovirus and *Salmonella* predominated in all age groups, and *Shigella* infections were found frequently in malnourished children. Future work on samples and data collected from this cohort

will be invaluable in providing a more detailed evaluation of pathogenicity, seroconversion and immune response to a variety of important pathogens causing diarrhoea in this urbanising, industrialising setting in Southeast Asia.

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Table 1: **Baseline characteristics of cohort members stratified by diarrhoeal pathogen, n(%) or median(interquartile range) as appropriate**

Characteristic	*C. difficile* n=36	*Campy* n=24	*Salmonella* n=44	*Shigella* n=9	Adenovirus n=25	Norovirus n=56	Rotavirus n=28	Total n=748
Median age in months	15.3 (14-19)	22.7 (16-28)	18.4 (15-24)	22.3 (18-31)	21.1 (17-25)	18.6 (15-25)	18.9 (17-22)	24.3 (12.6-30.4)
Male sex	27 (75.0%)	14 (58.3%)	28 (63.6%)	6 (66.7%)	14 (56%)	40 (71.4%)	20 (71.4%)	398 (53.2%)
Growth metrics at 12m†								
Weight for age	-0.08 (-0.7-0.7)	-0.13 (-0.5-0.6)	-0.05 (-0.6-0.7)	-0.33 (-0.5-0.3)	0.02 (-0.6-0.5)	0.08 (-0.4-0.6)	-0.05 (-0.7-0.8)	0.21 (-0.5 - 1.1)
Height for age	0.50 (-0.3-1.1)	0.01 (0.6-0.7)	0.31 (-0.3-0.8)	0.14 (0-0.9)	0.34 (-0.3-0.8)	0.38 (-0.4-0.7)	0.13 (-1.5)	-0.11 (-0.8 - 0.5)
Weight for height	-0.10 (-0.7-0.4)	0 (-0.5-0.3)	0.10 (-0.6-0.3)	0.10 (-0.6-0)	-0.09 (-0.7-0.4)	-0.10 (-0.5-0.5)	0 (-0.7-0.8)	0.39 (-0.38 - 1.2)
Maternal education‡								
Lower secondary	17 (47.2%)	10 (41.7%)	23 (52.3%)	5 (55.6%)	12 (48.0%)	35 (62.5%)	19 (67.9%)	394 (52.7%)
Higher secondary	19 (52.8%)	14 (58.3%)	21 (47.7%)	4 (44.4%)	13 (52.0%)	21 (37.5%)	9 (32.1%)	354 (47.3%)
Rotavirus vaccine								
1 dose	17 (47.2%)	9 (37.5%)	16 (36.4%)	4 (44.4%)	6 (24.0%)	25 (44.6%)	9 (32.1%)	314 (42.0%)
2 doses	15 (41.7%)	9 (37.5%)	16 (36.4%)	4 (44.4%)	6 (24.0%)	23 (41.1%)	9 (32.1%)	304 (40.6%)
Drinking water^								
Piped to home	24 (66.7%)	15 (62.5%)	28 (63.6%)	6 (66.7%)	14 (56.0%)	35 (62.5%)	16 (57.1%)	487 (65.1%)
Bottled	12 (33.3%)	9 (37.5%)	16 (36.4%)	3 (33.3%)	11 (44.0%)	20 (35.7%)	12 (42.9%)	255 (34.1%)
Toilet								
Toilet	6 (24.0%)	4 (23.5%)	4 (13.8%)	2 (28.6%)	4 (21.1%)	7 (17.1%)	6 (31.6%)	171 (26.7%)
Chamber pot	14 (56.0%)	10 (58.8%)	17 (58.6%)	3 (42.9%)	10 (52.6%)	28 (68.3%)	9 (47.4%)	400 (62.5%)
Floor	2 (8.0%)	2 (11.8%)	5 (17.2%)	2 (28.6%)	5 (26.3%)	3 (7.3%)	2 (10.5%)	55 (8.6%)
Other	3 (12.0%)	1 (5.9%)	3 (10.3%)	0 (0%)	0 (0%)	3 (7.3%)	2 (10.5%)	14 (1.9%)
Diaper	13 (36.1%)	8 (33.3%)	23 (52.3%)	3 (33.3%)	6 (24.0%)	22 (39.3%)	9 (32.1%)	126 (16.8%)
Household crowding*	22 (61.1%)	15 (62.5%)	30 (68.2%)	5 (55.6%)	12 (48.0%)	33 (58.9%)	15 (53.6%)	450 (60.6%)
Regular probiotics	11 (30.6%)	9 (37.5%)	20 (45.5%)	4 (44.4%)	12 (48.0%)	24 (42.9%)	16 (57.1%)	259 (34.6%)

† WHO Z-scores at 12 months of age; ‡ Lower secondary or below, higher secondary or above; ^One additional category "Other" for drinking water (not shown), Norovirus: 1 (1.8%) and Total: 6 (0.8%); *>2 people/room

Table 2: Count and incidence per 100 child years of follow up of pathogen specific diarrhoeal episodes diagnosed by either microbiological culture or the Luminex Gastrointestinal Pathogen Panel

Pathogen	Culture confirmed		Luminex	
	n	Incidence (95%CI)	n	Incidence (95%CI)
C. difficile			42	7.4 (4.8-9.9)
Campylobacter	16	2.6 (1.3-3.9)	24	4.1 (2.5-5.8)
E. coli O157			5	1.0 (0.1-1.9)
ETEC			28	4.5 (2.6-6.4)
Salmonella	9	1.51 (0.4-2.7)	54	8.8 (5.8-11.9)
Shigella	2	0.37 (0-0.9)	9	1.5 (0.5-2.6)
STEC			2	0.3 (0-0.7)
V. cholerae			0	0 (0)
Yersinia			0	0 (0)
Adenovirus			30	5.1 (2.9-7.3)
Norovirus			76	12.5 (8.4-16.5)
Rotavirus			29	4.8 (3.0-6.5)
Cryptosporidium	1	0.2 (0-0.5)	11	1.7 (0.7-2.8)
Giardia	0	0	3	0.4 (0-0.9)
E. histolytica	1	0.2 (0-0.7)	2	0.3 (0-0.8)

CI: confidence interval

Table 3: Number of episodes and incidence of diarrhoea per 100 child years by age group

Episodes	12-23 month		24-35 month		>=36 month	
	n	Incidence (95%CI)	n	Incidence (95%CI)	n	Incidence (95%CI)
Total	247	80.8 (63.9-97.7)	122	59.9 (32.8-87.0)	31	28.1 (15.2-41.0)
Home	122	40.8 (32.3-49.3)	83	29.4 (20.8-38.1)	22	19.3 (8.0-30.5)
Hospital	120	38.5 (25.6-51.3)	37	30.1 (4.3-55.9)	9	8.8 (2.5-15.1)
Luminex detection						
C. difficile	37	12.0 (7.6-16.5)	4	1.3 (0-2.6)	1	0.6 (0-1.8)
Campylobacter	14	4.2 (2.0-6.4)	6	14.1 (0-37.9)	4	3.1 (0-6.3)
E. coli O157	3	1.2 (0-2.5)	2	1.3 (0-3.5)	0	0 (0)
ETEC	19	5.8 (2.9-8.8)	7	3.3 (0.2-6.4)	2	2.0 (0-4.9)
Salmonella	40	13.0 (7.8-18.1)	14	17.9 (0-42.1)	0	0 (0)
Shigella	5	1.5 (0.2-2.8)	4	2.1 (0-4.5)	0	0 (0)
STEC	2	0.6 (0-1.4)	0	0 (0)	0	0 (0)
Adenovirus	19	6.2 (3.2-9.3)	9	5.4 (0.5-10.3)	2	2.6 (0-6.5)
Norovirus	54	16.7 (10.1-23.4)	22	9.0 (3.7-14.3)	0	0 (0)
Rotavirus	24	7.8 (4.5-11.0)	4	1.2 (0-2.4)	1	1.3 (0-3.7)
Cryptosporidium	9	3.1 (0.9-5.2)	2	0.6 (0-1.5)	0	0 (0)
Giardia	2	0.5 (0-1.2)	1	0.2 (0-0.6)	0	0 (0)
E. histolytica	0	0 (0)	2	0.6 (0-1.5)	0	0 (0)

CI: confidence interval

Table 4: Clinical characteristics of pathogen-specific monoinfections and broad classification of coinfection (bacterial, viral or bacterial/viral), n(%) or median (interquartile range) as appropriate

Characteristic	Monoinfection							Coinfection		
	C. difficile	***Campylobacter***	***Salmonella***	***Shigella***	**Adenovirus**	**Norovirus**	**Rotavirus**	**Bacterial**	**Viral**	**Bac/Viral**
	n=10	n=7	n=9	n=5	n=5	n=24	n=9	n=8	n=7	n=31
Type of diarrhoea										
Watery	7 (77.8%)	5 (71.4%)	7 (77.8%)	3 (60.0%)	5 (100%)	20 (83.3%)	9 (100%)	2 (25.0%)	6 (85.7%)	29 (93.5%)
Mucoid	4 (44.4%)	3 (42.9%)	5 (55.6%)	4 (80.0%)	2 (40.0%)	10 (41.7%)	3 (33.3%)	7 (87.5%)	5 (71.4%)	21 (67.7%)
Bloody	1 (11.1%)	2 (28.6%)	1 (11.1%)	1 (20.0%)	0 (0%)	1 (4.2%)	0 (0%)	2 (25.0%)	1 (14.3%)	2 (6.5%)
No episodes/24 hr	5 (3-6)	6 (2-10)	6 (5-8)	6 (6-10)	3 (3-7)	5 (4-7.5)	5 (4-8)	5.5 (5-6)	4 (3-4)	5 (4-7)
Temperature	37 (37-37)	37 (37-37.3)	37 (37-37.8)	38.5 (37.5-38.5)	37 (37-37)	37 (37-37)	38 (37-38)	37.3 (37-38)	38 (37-39.2)	37 (37-37.6)
Symptoms										
Vomit	5 (55.6%)	3 (42.9%)	5 (55.6%)	2 (40.0%)	2 (40.0%)	16 (66.7%)	7 (77.8%)	5 (62.5%)	5 (71.4%)	17 (54.8%)
Abdominal pain	3 (33.3%)	1 (14.3%)	3 (33.3%)	4 (80.0%)	3 (60.0%)	9 (37.5%)	1 (11.1%)	2 (25.0%)	3 (42.9%)	7 (22.6%)
Therapies										
Antimicrobials	2 (22.2%)	4 (57.1%)	6 (66.7%)	4 (80.0%)	2 (40.0%)	13 (54.2%)	4 (44.4%)	6 (75.0%)	6 (85.7%)	18 (58.1%)
Rehydration	4 (44.4%)	6 (85.7%)	6 (66.7%)	3 (60.0%)	5 (100%)	16 (66.7%)	6 (66.7%)	5 (62.5%)	6 (85.7%)	16 (51.6%)
Zinc	4 (44.4%)	3 (42.9%)	4 (44.4%)	3 (60.0%)	1 (20.0%)	11 (45.8%)	4 (44.4%)	6 (75.0%)	2 (28.6%)	12 (38.7%)
Probiotics	8 (88.9%)	7 (100%)	8 (88.9%)	3 (60.0%)	5 (100%)	23 (95.8%)	9 (100%)	8 (100%)	6 (85.7%)	27 (87.1%)
Haematology										
WBC $x10^9$/L	10.7 (8-13)	13.6 (10-16)	12.9 (10-16)	10.2 (10-15)	7.2 (7-7)	10.2 (8-12)	11.6 (9-16)	16.3 (11-21)	12.3 (12-16)	9.7 (8-12)
Neutrophils (%)	28.9 (22-40)	54.9 (39-72)	51.5 (41-55)	60.9 (55-63)	22.4 (19-26)	41.1 (32-59)	49.6 (39-64)	53.5 (43-59)	59.6 (56-62)	45 (32-52)
Lymphocytes (%)	52.4 (43-60)	33.7 (18-50)	35.4 (30-38)	32.4 (26-33)	64.9 (63-67)	40 (29-49)	36.4 (21-44)	38 (37-42)	28.5 (27-31)	40.9 (33-51)
Haematocrit (%)	39.3 (36-42)	35.7 (34-38)	32.9 (31-38)	37.3 (35-38)	35.3 (35-36)	36.6 (33-38)	34.7 (31-42)	36.3 (34-39)	35.4 (34-39)	35.5 (34-37)

WBC: white blood cell count

Table 5: Univariate logistic regression evaluating any diarrhoeal episode positive by Luminex against the remainder of the cohort (both Luminex-negative and diarrhoea-negative).

Characteristic	***C. difficile***		***Campylobacter***		***Salmonella***		***Shigella***		**Adenovirus**		**Norovirus**		**Rotavirus**	
	OR 95%CI	p	OR 95%CI	p	OR 95%CI	p	OR 95%CI	p	OR 95%CI	p	OR 95%CI	p	OR 95%CI	p
Age (month)	**0.87 0.81-0.93**	**<0.001**	1.02 0.96-1.07	0.546	**0.94 0.90-0.98**	**0.006**	1.02 0.94-1.11	0.569	0.99 0.95-1.04	0.784	**0.95 0.92-0.99**	**0.006**	0.97 0.92-1.02	0.205
Male sex	**2.76 1.28-5.94**	**0.010**	1.24 0.54-2.83	0.610	1.58 0.84-2.97	0.156	2.77 0.44-7.13	0.433	1.12 0.50-2.51	0.776	**2.33 1.28-4.24**	**0.006**	**2.26 0.98-5.20**	**0.055**
Growth metrics at 12m														
WAZ	1.11 0.96-1.27	0.150	1.10 0.91-1.34	0.314	1.02 0.88-1.17	0.826	**0.62 0.43-0.89**	**0.009**	**1.45 1.21-1.73**	**<0.001**	0.91 0.79-1.03	0.134	0.96 0.81-1.14	0.655
HAZ	1.10 0.97-1.25	0.131	0.98 0.82-1.17	0.815	1.02 0.90-1.17	0.715	**0.68 0.50-0.93**	**0.015**	**1.33 1.13-1.57**	**0.001**	0.90 0.80-1.01	0.081	**0.79 0.68-0.92**	**0.003**
WHZ	1.04 0.91-1.18	0.574	1.09 0.92-1.30	0.320	1.0 0.88-1.14	0.995	0.82 0.59-1.14	0.241	**1.20 1.02-1.41**	**0.03**	0.95 0.84-1.07	0.378	1.15 0.99-1.34	0.064
Low maternal education	0.80 0.41-1.55	0.503	0.63 0.28-1.44	0.276	0.98 0.53-1.81	0.956	1.12 0.30-4.22	0.862	0.82 0.37-1.83	0.635	1.55 0.88-2.71	0.128	1.94 0.87-4.35	0.107
Rotavirus vaccination	1.36 0.64-2.46	0.503	0.83 0.36-1.92	0.661	0.78 0.42-1.47	0.448	1.11 0.30-4.18	0.874	0.43 0.17-1.08	0.074	1.13 0.65-1.96	0.659	0.65 0.29-1.45	0.292
Toilet^														
Chamber pot	1.0 0.38-2.64	0.996	1.07 0.33-3.46	0.909	1.85 0.61-5.59	0.274	0.64 0.11-3.86	0.625	1.07 0.33-3.46	0.909	1.76 0.75-4.12	0.190	0.63 0.22-1.81	0.393
Floor	1.04 0.20-5.30	0.964	1.58 0.28-8.84	0.606	**4.18 1.08-16.1**	**0.038**	3.19 0.44-23.2	0.252	**4.18 1.08-16.1**	**0.038**	1.35 0.34-5.42	0.670	1.04 0.20-5.30	0.964
Diaper*	1.48 0.71-3.1	0.291	1.84 0.73-4.67	0.198	**3.68 1.9-7.12**	**<0.001**	1.72 0.39-2.57	0.476	0.87 0.33-2.28	0.772	**2.13 1.16-3.90**	**0.015**	1.27 0.55-2.96	0.578

Household crowding†	1.02 0.52-2.04	0.945	1.09 0.47-2.52	0.844	1.42 0.74-2.73	0.289	9.81 0.22-3.04	0.757	0.59 0.27-1.31	0.196	0.93 0.53-1.62	0.794	0.74 0.25-1.58	0.442
Regular probiotics	0.82 0.40-1.70	0.599	1.14 0.49-2.64	0.764	1.62 0.88-2.99	0.123	1.52 0.40-5.70	0.536	1.78 0.80-3.96	0.158	1.46 0.84-2.53	0.180	**2.62** **1.22-5.62**	**0.014**

* controlled by age; ^Flush toilet as the reference category; † >2 people/room; OR: odds ratio; CI: confidence interval; WAZ: weight for age Z-score; HAZ: height for age Z-score; WHZ: weight for height Z-score

Figure 1: Pathogen specific incidence of a selection of aetiologies detected by the Luminex Gastrointestinal Pathogen Panel across age groups. Top row: incidence of ten pathogens per 100 child years of follow up in 12-23 months, 24-35 months and ≥36 months of age. Bottom row: proportion of diarrhoeal stools that were positive for each pathogen, 12-23 months, 24-35 months and ≥36 months of age. C diff: *Clostridium difficile*; Campy: *Campylobacter* spp.; O157: *E. coli* O157; ETEC: Enterotoxigenic *E. coli*; Salm: *Salmonella* spp.; Shig: *Shigella* spp.; Adeno: adenovirus; Noro: norovirus; Rota: rotavirus; Crypto: *Cryptosporidium.*

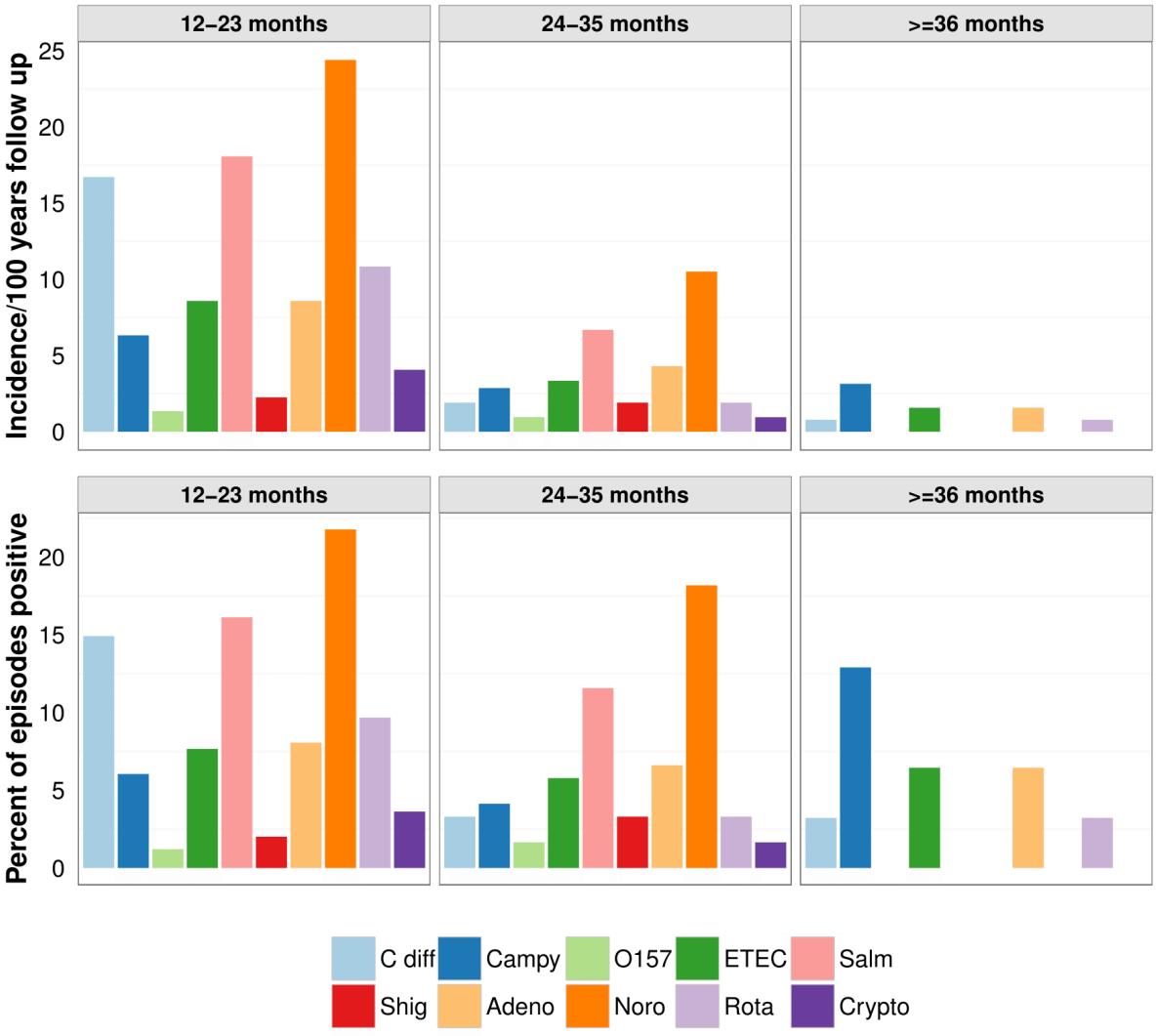


Figure 2: Coinfection matrix. Each detected pathogen is displayed in matrix format. The color represents the correlation of coinfection between both pathogens: the darker the red, the more positive the Pearson correlation coefficient and the darker the blue, the more negative the Pearson correlation coeffficient. C diff: *Clostridium difficile*; Campy: *Campylobacter* spp.; O157: *E. coli* O157; ETEC: Enterotoxigenic *E. coli*; Salm: *Salmonella* spp.; Shig: *Shigella* spp.; STEC: Shiga-toxin producing *E. coli;* Adeno: adenovirus; Noro: norovirus; Rota: rotavirus; Crypto: *Cryptosporidium;* Ent Hist: *Entamoeba histolytica.*

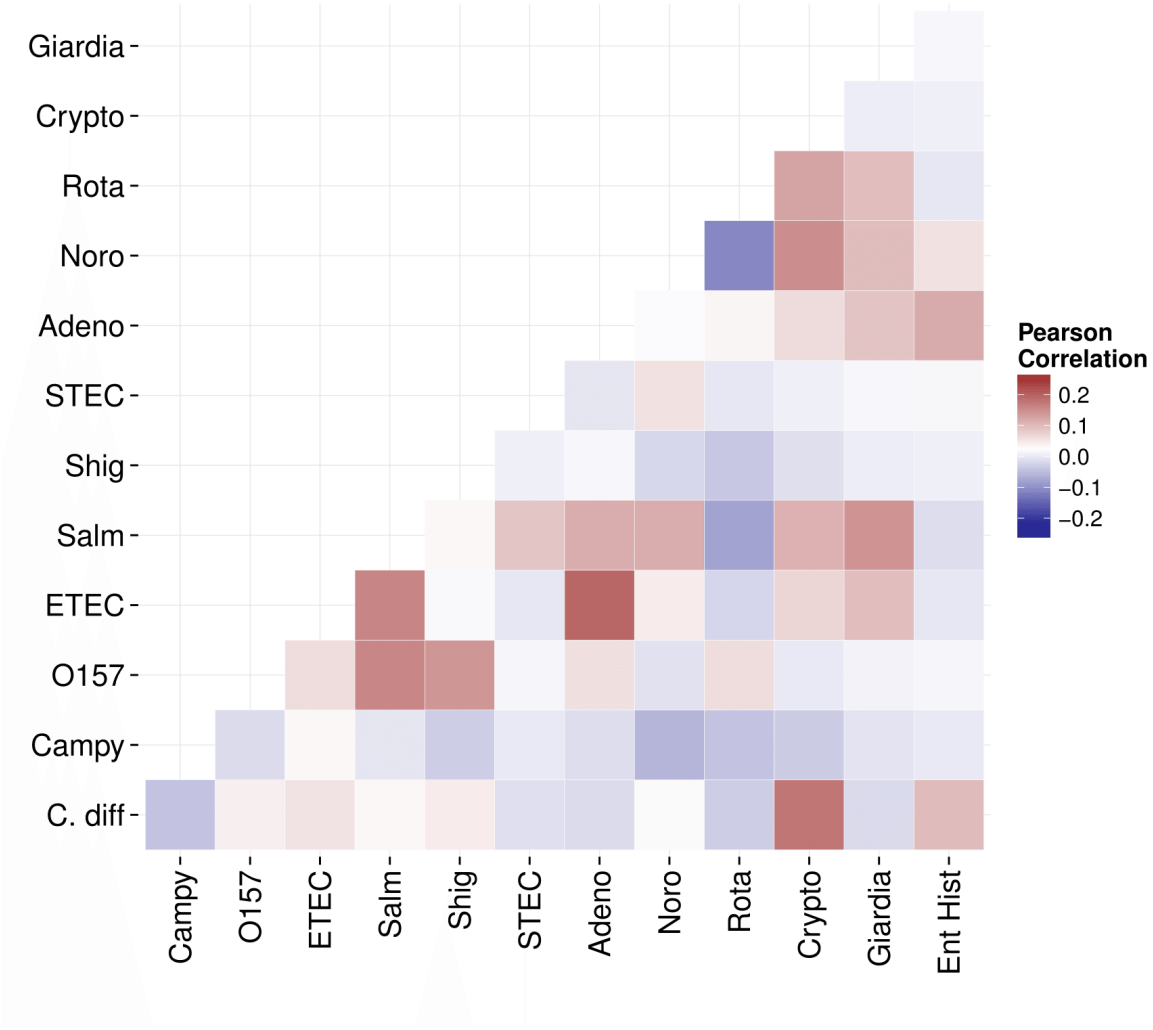
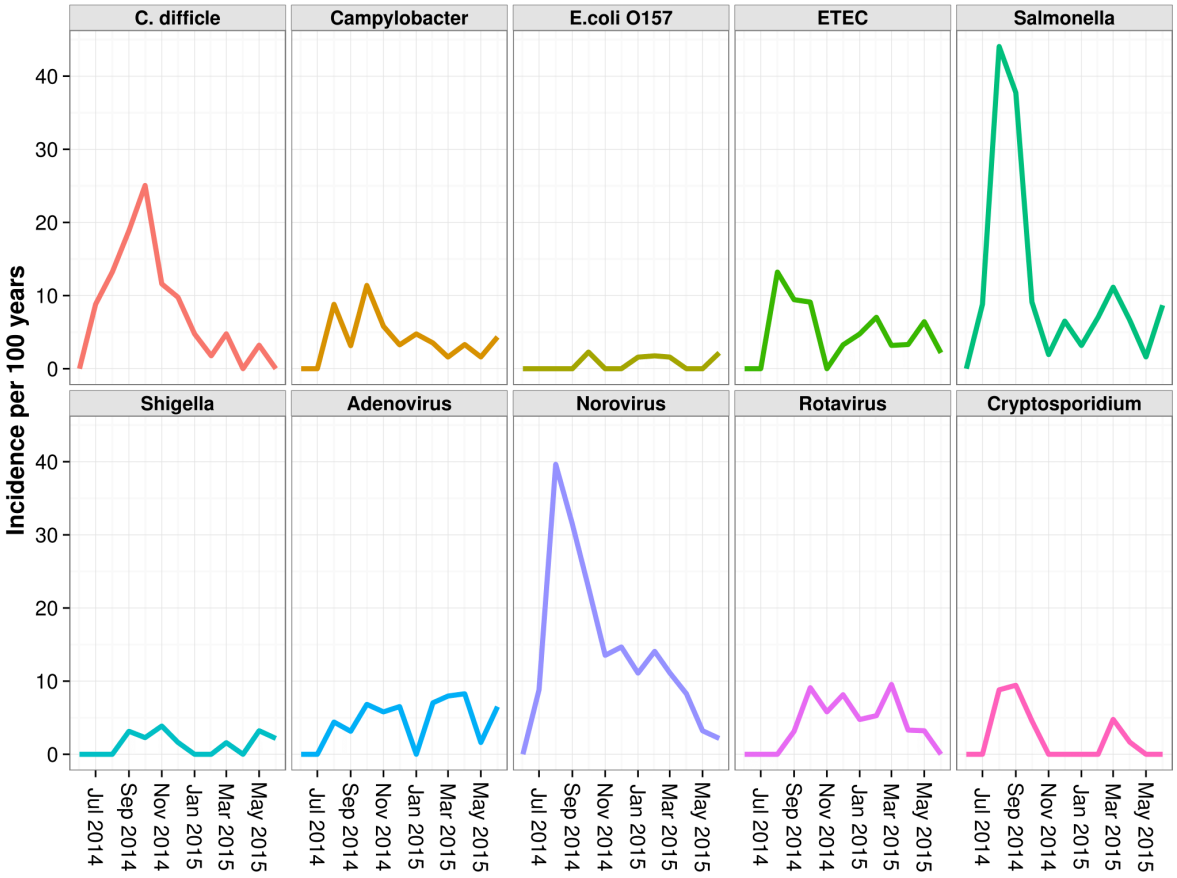


Figure 3: Seasonality of incidence of selected pathogens. The monthly incidence per 100 child years of follow up of each pathogen is shown in a different panel and in a different color, with pathogen names at the top of each plot.



9 RESEARCH PAPER 7: The transfer and decay of maternal antibody against *Shigella sonnei* in a longitudinal cohort of Vietnamese infants

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SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection and disease in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Vaccine
Please list the paper's authors in the intended authorship order:	Corinne N. Thompson, Le Thi Phuong Tu, Katherine L. Anders, Nguyen Trong Hieu, Lu Lan Vi, Nguyen Van Vinh Chau, Vu Thuy Duong, Nguyen Ngoc Minh Chau, Tran Thi Hong Chau , Ha Thanh Tuyen, Tran Vu Thieu Nga, Pham Van Minh, Tran Do Hoang Nhu, Le Thi Quynh Nhi, Allan Saul, Laura B. Martin, Audino Podda, Christiane Gerke, Guy Thwaites, Cameron P. Simmons and Stephen Baker
Stage of publication	**In press**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am first author. I led the management of the cohort study, oversaw data collection and management and cleaned all data for this manuscript. I developed the analysis and code in STATA and R, with assistance from Dr Phung Khanh Lam in the OUCRU Biostatistics Department. I made all tables and figures, wrote the manuscript and was responsible for submisison of the work.

Student Signature: _____ **Date:** 16 NOV 2015

Supervisor Signature: _____ **Date:** 16/11/15

Title: The transfer and decay of maternal antibody against *Shigella sonnei* in a longitudinal cohort of Vietnamese infants

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Running title: *Shigella* antibody dynamics in infancy

Key words: Shigella, maternal antibody, placental transfer; seroconversion

Abstract

Background: *Shigella sonnei* is an emergent and major diarrheal pathogen for which there is currently no vaccine. We aimed to quantify duration of maternal antibody against *S. sonnei* and investigate transplacental IgG transfer in a birth cohort in southern Vietnam.

Methods and Results: Over 500-paired maternal/infant plasma samples were evaluated for presence of anti-*S. sonnei*-O IgG and IgM. Longitudinal plasma samples allowed for the estimation of the median half-life of maternal anti-*S. sonnei*-O IgG, which was 43 days (95% confidence interval: 41-45 days). Additionally, half of infants lacked a detectable titer by 19 weeks of age. Lower cord titers were associated with greater increases in *S. sonnei* IgG over the first year of life, and the incidence of *S. sonnei* seroconversion was estimated to be 4/100 infant years. Maternal IgG titer, the ratio of antibody transfer, the season of birth and gestational age were significantly associated with cord titer.

Conclusions: Maternal anti-*S. sonnei*-O IgG is efficiently transferred across the placenta and anti-*S. sonnei*-O maternal IgG declines rapidly after birth and is undetectable after five months in the majority of children. Preterm neonates and children born to mothers with low IgG titers have lower cord titers and therefore may be at greater risk of serconversion in infancy.

INTRODUCTION

The bacterial genus *Shigella* is a major contributor to the global burden of diarrheal disease. This genus of enteric pathogens is typically associated with disease in children under five years of age in industrializing regions (1), and is estimated to be responsible for 100,000 deaths annually (2). *Shigella* infections are characteristically associated with dysentery (blood and mucus in the stool) and can be severe in young children (3, 4). Of the four *Shigella* species, *S. flexneri* and *S. sonnei* predominate worldwide (1). *S. flexneri* is traditionally associated with disease in industrializing countries, whereas *S. sonnei* is more commonly isolated in industrialized regions. However, this distribution is changing. *S. sonnei* is globally emergent and replacing *S. flexneri* as the most common cause of bacterial dysentery (5, 6). This trend may be being exacerbated by resistance to common antimicrobials, with several recent reports of *S. sonnei* exhibiting resistance against fluoroquinolones and 3rd generation cephalosporins in the USA, Vietnam and elsewhere (7–9). Improved sanitation and antimicrobial treatment remain the only current tools for prevention and control as there are no licensed *Shigella* vaccines (10).

Neonates and infants are typically at increased risk from infectious agents such as *Shigella* due to immaturity of the immune system (11). While neonates have some capacity for cell-mediated immunity (12), humoral immunity is very limited in early life (13). Antibody responses in neonates are shorter, delayed in onset and of lower affinity than those observed in healthy adults (14). The transfer of maternal IgG antibody to the fetus during pregnancy confers short-term passive immunity and represents a primary mechanism for protection against infectious diseases at birth (11). Transport of maternal antibody across the placenta to fetal capillaries is mediated by the neonatal Fc receptor

(FcRn) (15–17) and can be affected by factors such as gestational age, maternal IgG concentration and infection (18–21).

Maternally transferred IgG against *S. sonnei* in infancy has not been substantially investigated. Work conducted in Israel in the mid-1990s found that the concentration of anti-*S. sonnei* lipopolysaccharide (LPS) IgG present in umbilical cord plasma positively correlated with the concentration in maternal plasma (22). IgG against LPS, specifically the O-antigen component, is the best described *S. sonnei* immune marker as it is the major bacterial surface antigen exposed to the immune system during infection. Although anti-*S. sonnei*-O IgG is not a definitive correlate of protective immunity (23), it is an indicator of some degree of acquired immunity; lack of *Shigella* serotype specific antibody is associated with an increased risk of symptomatic disease (24, 25). Furthermore, titers of anti-*S. sonnei*-O IgG rise significantly after symptomatic infection (22, 26, 27), with titers doubling ten weeks post-infection (26, 28). Previous work from Vietnam in the late 1980s showed that anti-*S. sonnei*-LPS and anti-*S. flexneri*-LPS IgG rise dramatically from birth, peak at 3-4 years of age and then permanently plateau (29).

An understanding of the nature and duration of maternal antibody protection in infancy is important for determination of an appropriate vaccination schedule when *Shigella* vaccines eventually become available. Additionally, although IgG titers against *S. flexneri* and *S. dysenteriae* type I in Vietnam were found to be high in children and adults in the early 1990s (27, 29), exposure to *Shigella* has not been measured in a contemporary Vietnamese population. As *S. sonnei* is now the predominant *Shigella* species in Vietnam (30), we hypothesized there would be substantial evidence of population exposure and *S. sonnei* maternal antibody transfer in this rapidly industrializing country. Therefore, we

aimed to quantify maternal anti-*S. sonnei*-O antibody decay using the largest sample size to date, with over 500 paired mother and infant plasma samples. We also investigated transplacental IgG transfer and determined the incidence of *S. sonnei* seroconversion in infancy in southern, urban Vietnam.

METHODS

Ethical approval

Written informed consent was required from all enrolled families. Ethical approval was granted from Hung Vuong Hospital, Oxford Tropical Research Committee as well as the London School of Hygiene & Tropical Medicine for the main cohort study. Ethical approval was also granted from the Hospital for Tropical Diseases in HCMC and OxTREC for the studies collecting acute and convalescent plasma samples from culture-positive *Shigella* and *Salmonella* cases for ELISA validation.

Study population

The birth cohort population and methodology has been described previously in detail (31). Briefly, mothers delivering at Hung Vuong obstetric hospital in Ho Chi Minh City (HCMC) were invited to enroll during either an antenatal visit in the final month of pregnancy or at the time of hospital admission for delivery. Children born between January and December 2013 in HCMC were included in the analysis presented here. Pregnant women were eligible if they lived in district 8 of HCMC (a previously identified endemic hotspot for *Shigella* (30)), were aged 16 years or older and were HIV seronegative at the time of birth. Mothers answered a baseline questionnaire and blood (umbilical cord and venous) samples were collected in EDTA tubes. After delivery, infants were recalled regularly for routine follow up visits. A 1ml EDTA blood sample

was collected at the 4, 9 and 12 month visits. All blood samples were separated into cells and plasma and stored at -20^{o}C until required.

S. sonnei anti-O antigen ELISA

Antibody (IgG and IgM) against *S. sonnei* O-antigen were measured using an enzyme-linked immunosorbent assay (ELISA) in maternal, umbilical and longitudinally collected infant plasma samples. Purified *S. sonnei* O-antigen was extracted as previously described (32) and provided by Sclavo Behring Vaccines Institute for Global Health (Siena, Italy). For the ELISA assays, 96-well microtiter plates (Maxisorb; NUNC) were coated overnight with 0.5mg/mL *S. sonnei* O-antigen in PBS pH 7.0 at 4^{o}C, plates were then washed and blocked in PBS containing 5% skimmed milk powder for 2 hours. After washing, 100µl of each plasma sample (diluted at 1:200 in PBS containing 1% skimmed milk) were added and plates were incubated for 2 hours at room temperature. IgG and IgM against *S. sonnei* O-antigen were detected by incubation with alkaline phosphatase directly conjugated anti-human IgG/IgM for 1 hour. Plates were developed by p-nitrophenyl-phosphate solution (Sigma) and were read at absorbance 405nm and 490nm by an ELISA platereader (Microplate reader, Biorad). Each plate contained a 2-fold serially diluted pool of anti- *S. sonnei*-O antigen human plasma (primary concentration 1:200). A standard curve was generated from the corresponding optical density (OD) and ELISA units using a 4-parameter logistic regression fit. One ELISA unit (EU) was defined as the reciprocal dilution of the standard plasma that gave an absorbance value equal to 1 in this assay. The ELISAs were done in duplicate. Antibody (IgG and IgM) units in the cohort members' plasma were calculated relative to this standard each time the assay was performed. Acute and convalescent plasma samples for the ELISA validation were derived from pediatric culture-positive *S. sonnei* and *Salmonella*

dysentery cases presenting to either the Hospital for Tropical Disease in HCMC as part of another ongoing study.

Statistical analyses

Geometric mean titers (GMT) were calculated to summarize anti-*S. sonnei*-O IgG in maternal and cord plasma. Paired t-tests were used to compare $\log_{10}$ titers between paired maternal/cord samples. Analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was used to compare maternal and cord log-transformed antibody titers within categorical groups. The ratio of maternal transfer was compared across groups using the Kruskal-Wallis (KW) test with Dunn's test for multiple comparisons (33). Linear mixed effects modeling was used to assess the trajectory of infant $\log_{10}$ titers from birth to 20 weeks to account for within-participant association over time. The half-life of IgG titer was calculated as the time at which the predicted IgG titer would decrease by 50% from the cord blood titer. The population half-life was derived using the formula:

$$\frac{-\log_{10}(2)}{b1}$$

with $b1$ equal to the slope of the fixed effect. The 95% confidence interval (CI) for the population level half-life was derived from the CI of the slope of the fixed effect. Children with a 4-fold rise between serial titers or those who were aged <6 months without a decrease in IgG titer were censored after the time point prior to the increase or no decrease, respectively (34).

A Kaplan Meier survival curve was generated to investigate the time taken for titers to fall below a detectable threshold of 10.3EU. This threshold value was determined by calculating the mean titer value of the observation preceding a 4-fold rise in titer in infants that had a 4-fold rise with a gap between pre and post-seroconversion samples no

greater than 24 weeks (n=15). None of the cord plasma titers and less than 1% (2/502) of the maternal plasma samples had titers that fell below 10.3EU. For the Kaplan Meier estimation, infants were censored either when they (1) dropped below 10.3EU (2) had any rise in IgG titer or (3) were lost to follow up. Finally, linear regression was used to evaluate the effect of covariates on anti-*S. sonnei*-O IgG cord titer as well as the relationship between $\log_{10}$ cord titer and $\log_{10}$ increase in titer between serial follow up visits. All analyses were performed in STATA v13 (TX, USA) with the exception of the mixed effects modeling which was performed in R (version 3.0.2) using the lme4 package (35). Plots were made in R using the ggplot package v1.0.1 (36).

RESULTS

ELISA validation

We firstly validated the anti-*S. sonnei*-O ELISA in a population of Vietnamese children hospitalized with dysentery with acute and convalescent plasma samples. All tested (7/7; 100%) stool culture-positive *S. sonnei* cases presenting to hospital had >4 fold rise (median: 104-fold; range: 22-410) in IgG titer regardless of the number of days between the acute and convalescent samples (median: 116 days; range: 13-202). The IgM titers against *S. sonnei* O-antigen of the seven responding children also increased dramatically (median: 9-fold, range:3-64). Twenty culture positive *Salmonella* cases from the same study did not generate an *S. sonnei* O-antigen IgG response (median fold titer increase: 1.1, range: 0-2.0), with limited IgM response as well (median: 1.4-fold, range: 0-58) (data not shown).

Cohort baseline characteristics

Of the 503 infants enrolled into the birth cohort in 2013, 52% (260/503) were male, 4% (21/503) were born preterm (<37 weeks of gestation) and 5% (23/503) were of low birth weight (<2.5kg) as shown in Table 1. The median maternal age was 28 years (interquartile range (IQR): 25-31), with just under half of all mothers (244/503; 49%) reporting at least a higher secondary education. The median maternal gravidity was 2 (IQR: 1-3) and the mean duration of infant follow up was 337 days (range: 1-399 days). A total of 58% (292/503) infants enrolled returned for all three follow up appointments where a blood sample was collected (Figure 1A). A further 78% (393/503) returned for at least two blood-draw appointments, and 86% (432/503) for at least one follow up blood-draw appointment. There were no major demographic or socioeconomic differences between the families of infants who did not return for all four follow up visits (211/503; 42%) and those that did return for all four visits (292/503; 58%) (Table 2).

The decay of maternal anti-*S. sonnei*-O IgG and incidence of seroconversion

The anti-*S. sonnei*-O IgG and IgM titers in infants over the first 12 months of life are shown in Figures 1B and 1C, respectively. Using samples collected within 20 weeks of birth, we estimated the median half-life of anti-*S. sonnei*-O IgG to be 43.2 days (95%CI: 41.9 – 44.5 days). As shown in Figure 2, by 18.7 weeks (95%CI: 18.1-20.1 weeks) 50% of infants had undetectable levels of anti-*S. sonnei*-O IgG. A total of 16 children had a >4-fold rise in anti-*S. sonnei*-O IgG titer in the first year after birth (3.2%), the majority of which occurred between 4-9 months (8/16, 50%), or 9-12 months (6/16, 38%) after birth (Figure 2). Critically, a higher fold-rise in anti-*S. sonnei*-O IgG over the first 12 months of life was associated with a lower cord titer (p<0.001; linear regression). There were 463.5 infant years of follow up in this cohort, leading to a seroconversion rate (defined by >4-fold rise in titer) of 3.5/100 years of follow up in the first 12 months of

life. Two children did not have a detectable decrease in IgG titer between birth and 20 weeks of life (0.4%). Furthermore, 49/503 infants (10%) had a 2-fold rise in anti-*S. sonnei*-O IgG over the course of the first 12 months of life, the majority of which (25/49, 51%) occurred between 9-12 months of age. Out of the 503 infants enrolled in the cohort, 162 (32%) had a rise (any) in titer over the first year of life, which were more commonly detected between 9-12 months of age (84/162; 52%).

Maternal antibody transfer

The geometric mean titers of anti-*S. sonnei*-O IgG in cord plasma and maternal plasma were 234.1EU (range: 21.6-3,687.6EU) and 167.4EU (range: 3.75-2,553EU), respectively (Table 2). The median ratio of cord:maternal plasma anti-*S. sonnei*-O IgG was 1.32 (range: 0.3-12.4) (Table 2). Anti-*S. sonnei*-O IgG titers in cord plasma were consistently and significantly higher than those in maternal plasma (Table 2), with the exception of babies born preterm (p=0.71, paired t-test of $\log_{10}$ titers). The ratio of maternal transfer in preterm babies (median: 1.13) was significantly lower than in babies born 37-40 weeks (median: 1.35) (p=0.02; KW). Furthermore, the transplacental transfer ratio in first-pregnancy mothers (median: 1.38) was moderately higher than in mothers that had had previous pregnancies (median: 1.30, p=0.066; KW). The maternal transfer ratio was also slightly greater in younger mothers (<28 years) than in mothers ≥28 years of age (median: 1.36 versus 1.29, respectively; p=0.065; KW). Finally, the transplacental transfer ratio was significantly higher in infants born in January – March (median: 1.70) and in April – June (median: 1.90) compared to those born in July – September (median: 1.14) and October – December (median: 1.12) (Table 3).

Factors influencing anti-S. sonnei-O cord blood antibody titers

Anti-*S. sonnei*-O cord IgG titer was associated with several covariates in a univariate analysis (Table 3). However, after controlling for the effects of confounding in an adjusted analysis the only covariates that remained significantly associated with anti-*S. sonnei*-O cord IgG titer were ratio of transplacental IgG transer, maternal IgG titer and the season of birth. As shown in Figure 3A, anti-*S. sonnei*-O cord IgG titers in babies born in the first half of the year were higher than those born in the second half of the year. The ratio of anti-*S. sonnei*-O IgG maternal transfer was also elevated in the first half of the year compared to the later months (Figure 3B). Significantly, the ratio of transplacental transfer was higher in mothers with low IgG at the time of birth (Figure 3C) ($p<0.001$; linear regression of $\log_{10}$ maternal titers).

On additional analysis we found that maternal IgM increased (Figure 3D) from May to July, plateauing in the latter months of the year, suggesting that some mothers were likely exposed to *S. sonnei* at the time of birth during April-June. Maternal IgG levels did not significantly change throughout the year, although the titers were generally high, suggesting previous and potentially sustained exposure. The combination of low existing maternal IgG in some mothers during April – June and the increased the ratio of transplacental transfer during this period lead to an overall elevated anti-*S. sonnei*-O IgG in babies born during this period, which may be during a period of increased seasonal *S. sonnei* transmission in HCMC.

DISCUSSION

S. sonnei is an emergent and increasingly antimicrobial resistant diarrheal pathogen. As such *S. sonnei* is a growing challenge in Vietnam and other similarly industrializing countries (5, 6, 37–42). The aims of this study were: 1) to quantify the duration of

maternal IgG in infants, 2) to measure incidence of *S. sonnei* seroconversion in the first year of life and 3) to examine transplacental IgG transfer during pregnancy. As *S. sonnei* vaccines are in development (23), understanding the potential of maternal immunity in infants will be critical for evaluating future vaccine efficacy and identifying the infant groups that are most at risk of *S. sonnei* seroconversion (4).

The estimated half-life of maternal anti-*S. sonnei*-O IgG (43 days, 95%CI: 42-45 days) is similar to that of *Haemophilus influenza*e (33 days), pertussis (36-40 days) and *S. pneuomoniae* (35 days) (43–45). However, as the sampling was infrequent in the early weeks after birth these data should be interpreted with caution. Nevertheless, it is apparent that maternal antibody wanes rapidly and by five months of age the majority of infants had no circulating maternal antibody and are likely at increased risk of infection. Correspondingly, evidence of *S. sonnei* exposure in infants in our cohort suggests an incidence of seroconversion of approximately 4/100 infant years of follow up in HCMC. Yet given the known lack of general humoral immune responses against polysaccharides during infancy (13), in addition to loss to follow up, this seroconversion incidence is likely an underestimate.

We found that lower cord titers were associated with higher fold-increases in anti-*S. sonnei*-O IgG titer in the first year of life in our cohort, suggesting that neonates born with lower cord titers are at increased risk of seroconversion during infancy. The most important influences on anti-*S. sonnei*-O cord titer were maternal IgG titer and the ratio of transplacental transfer, which were inversely correlated. Such a relationship is due in part to saturation of the Fc receptor, as IgG that is not bound is digested by lysosomal enzymes inside the syncytiotrophoblast (11, 46). The negative relationship between

maternal IgG concentration and transplacental transfer ratio has been suggested to demonstrate the existence of a mechanism to protect the newborn through strengthening the transfer of antibody when maternal levels are not optimally protective (47, 48). Furthermore, it has been found that a higher total maternal IgG concentration may lead to reduced transfer efficiency of both total and specific IgG (21), with some suggestion of receptor competition among antigen-specific IgG for the limited number of placental Fc receptors available (20).

Neonates tended to have elevated anti-*S. sonnei*-O IgG titers compared to mothers in our cohort. Such a phenomenon has been reported for a variety of pathogens including *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* (18, 47, 49). However, neonates born preterm in this cohort did not have an increased anti-*S. sonnei*-O titer relative to their mothers. As the majority of IgG is acquired by the fetus during the last four weeks of pregnancy (50), it follows that preterm neonates would lack maternal immunity and are potentially at increased risk for infections in the first few months of life. Furthermore, we found that children born to mothers with lower IgG titers had lower cord titers themselves and are at increased risk of exposure.

Interestingly, we noted a seasonal pattern to both cord plasma titers as well as the ratio of transplacental transfer in our cohort. Cord titers and the transplacental transfer ratio were higher in the second quarter of the year. Given the inverse relationship between maternal IgG titer and transfer ratio, we propose that this period may represent a time of increased transmission and, therefore, exposure to *S. sonnei* in HCMC. This hypothesis was supported by the observed increase in maternal IgM titer between May and July (representing acute infection), suggesting that mothers' existing immune response may be

naturally boosted during this time. If *S. sonnei* transmission in HCMC is more common between April and June then infants born in during this time are likely better equipped against *S. sonnei* exposure at birth as the cord titers are highest during this season. However, annual trends are difficult to evaluate from our yearlong dataset.

There were several limitations with this study. Firstly, the infrequent early blood samples from infants prevented high-resolution temporal analyses regarding maternal half-life duration and survival analysis of the waning of maternal IgG. Next, the lack of a similar cohort from a non-endemic area limits our ability to fully interpret the serology data in an epidemiological context. Furthermore, a lack of disease data prohibits an analysis of the protective effect of presence of antibody as well as a more detailed analysis of anti-*S. sonnei*-O IgG and IgM response in infants after infection. However, the major strength of this study is the cohort design and relatively limited loss to follow up which enables us to generalize our conclusions to Vietnamese infants in urban HCMC. In the future, investigations into additional protective factors against *Shigella*, such as breastfeeding, may be warranted (51).

In summary, *S. sonnei* exposure is common in HCMC and maternal IgG is readily transferred across the placenta, waning by 5 months of age in the majority of infants. In the event of licensure of a sufficiently safe and immunogenic *S. sonnei* vaccine, it would be prudent to vaccinate after the waning of maternal IgG in settings such as HCMC. Furthermore, we found that neonates have a higher concentration of IgG compared to mothers in most cases, and the ratio of transplacental transfer is inversely related to the maternal anti-*S. sonnei*-O IgG titer. Finally, we identified those likely to be more at risk of *S. sonnei* exposure in infancy to include preterm neonates and those born to mothers

with lower IgG titers. Therefore, appropriate monitoring and prevention strategies can be targeted to such groups.

Conflict of Interest

AS, LBM and AP are employed by Sclavo Behring Vaccines Institute for Global Health S.R.I, a GSK. All other authors declare no conflict of interest.

Author's contributions

CNT, KLA, LTQN, CS and SB designed and set up the cohort study. LTPT performed the ELISAs. CT carried out the analysis. NTH, LLV, NVVC, VTD, NNMC, TTHC, HHT, TVTN, PVM and TDHN were involved in the laboratory and clinical management of the cohort study. AS, LBM, AP and CG provided *S. sonnei* antigen and protocols for the ELISA. CT, GT and SB wrote the manuscript.

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Table 1: Baseline characteristics of 503 Vietnamese infants enrolled in the birth cohort in 2013

Characteristic	**n (%), median (IQR)**
Male sex	260 (51.7)
Gestational age (wks)	39 (38-40)
Preterm (<37 wks)	21 (4.2)
Birth weight (kg)	3.15 (2.9-3.4)
Low birth weight (<2.5kg)	23 (4.6)
Vaginal delivery	288 (57.3)
Breastfed during month 1	
Exclusively	215 (43.0)
Plus formula	243 (48.6)
No, only formula	42 (8.4)
Gravidity	2 (1-3)
Maternal education	
Lower secondary or below	255 (50.7)
Higher secondary or above	248 (49.3)
Maternal age (years)	28 (25-31)

Table 2: Demographic and socioeconomic characteristics of infants with plasma samples available from four follow up visits (0, 4, 9 and 12 months of age) and those who attended less than four visits, n(%)

Characteristic	**<4 visits** n=211	**4 visits** n=292	**p**[a]
Vaginal birth	111 (52.6)	177 (60.6)	0.073
Male infant	113 (53.6)	147 (50.3)	0.477
Infant low birthweight	9 (4.3)	14 (4.8)	0.779
Any previous children	128 (60.7)	185 (63.4)	0.539
Maternal age ≥28 years	109 (51.7)	150 (51.4)	0.949
Low maternal education	106 (50.2)	142 (48.6)	0.722
Household crowding	116 (55)	185 (63.4)	0.059
Infant cord $\log_{10}$ titer > 2.3^	122 (57.8)	151 (51.7)	0.175
Preterm (<37 wks)	9 (4.3)	12 (4.1)	1.00
Breastfed during month 1			
Exclusively	89 (42.6)	126 (43.3)	0.726
Plus formula	100 (47.8)	143 (49.1)	
Formula + food	20 (9.6)	22 (7.6)	
Mother ethnic minority	21 (10)	21 (7.2)	0.269
Father ethnic minority	26 (12.3)	24 (8.2)	0.129
Watersource			
Piped home	144 (68.2)	208 (71.2)	0.73
Bottled	63 (29.9)	80 (27.4)	
Other	4 (1.9)	4 (1.4)	

[a]p-value derived from chi-square or Fisher's exact test, ^median

Table 3: Geometric mean titers (GMT) of anti-*S. sonnei*-O IgG in maternal and cord plasma and the ratio of cord:maternal IgG titer

Category	n pairs	Maternal IgG GMT (range)	Cord IgG GMT (range)	Median ratio (range)	Comparison [a] p value	group
Total	503	167.4 (3.75-2553.7)	230.8 (0.22-3687.6)	1.33 (0-12.4)		
Gestational age						
<37 weeks (1)	21	190.8 (48.5-545.8)	197.4 (49.5-546.8)	1.13 (0.4-2.6)	0.019	1:2
37-40 weeks (2)	549	166.5 (3.7-2553.7)	232.1 (0.22-3687.6)	1.35 (0-12.4)	0.130	1:3
>40 weeks (3)	23	165.7 (22.3-1175.0)	237.7 (68.8-1163.3)	1.33 (0.6-6.2)	1.000	2:3
Sex[b]						
Female	243	149.2 (8.2-2553.7)	209.9 (0.22-2140.9)	1.37 (0-12.4)	0.177	
Male	260	186.4 (3.7-2524.2)	252.1 (23.1-3687.6)	1.28 (0.3-11.2)		
Birthweight						
<2500g	23	150.8 (26.1-1280.1)	195.9 (42.4-2140.9)	1.25 (0.5-3.8)	0.515	
≥2500g	480	168.3 (3.7-2553.7)	232.6 (0.22-3687.8)	1.33 (0-12.4)		
Gravidity						
1	190	150.5 (3.7-2553.7)	216.7 (0.2-2824.8)	1.39 (0-12.4)	0.066	
>1	313	178.6 (15.7-2524.2)	239.7 (23.1-3687.6)	1.3 (0.3-11.2)		
Maternal age [b,c]						
<28 years	244	138.7 (3.7-1530.9)	204.5 (1.1-2824.8)	1.36 (0.3-12.4)	0.065	
≥28 years	259	199.9 (20.6-2553.7)	258.6 (0.22-3687.6)	1.29 (0-8.3)		

Maternal education						
Lower secondary or below	255	179.9 (3.7-2553.7)	250 (26.7-3687.6)	1.29 (0.3-12.4)	0.449	
Higher secondary or above	248	155.5 (15.7-2524.2)	212.5 (0.22-3206.5)	1.35 (0-11.2)		
Maternal IgM [c]						
≤1.37	253	158 (3.7-2553.7)	268.4 (22.1-2824.8)	1.66 (0.37-12.4)	<0.001	
>1.37	250	177.5 (15.7-2524.2)	203.8 (21.6-3687.6)	1.16 (0.3-11.2)		
Season [c]						
Jan-Mar (1)	89	148.2 (3.7-2553.7)	244.4 (34.1-2824.8)	1.7 (0.6-11.8)	0.024	1:2
Apr-Jun (2)	133	174.3 (8.2-1596.0)	358.3 (65.3-3687.6)	1.9 (0.4-12.4)	<0.001	1:3
Jul-Sep (3)	161	162.6 (15.7-2524.2)	181.8 (22.1-3206.5)	1.14 (0.3-11.2)	<0.001	1:4
Oct-Dec (4)	120	182.3 (33.5-1969.7)	187 (0.22-2123.2)	1.12 (0-5.8)	<0.001	2:3
					<0.001	2:4
					1.000	3:4

[a] p-values comparing ratio of transfer between categories of each characteristic, p-values corrected for multiple comparisons are shown with groups indicated in parentheses next to the group name ; [b] significant difference in $\log_{10}$ titers of maternal plasma per category; [c] significant difference between $\log_{10}$ titers of cord samples per category ; titers are shown in ELISA Units (EU)

Table 4: Univariate and multiple linear regression measuring the effect of different covariates on the outcome of log_{10} cord anti-*S. sonnei*-O IgG titer

Characteristic	Cord blood IgG titer			
	univariate		adjusted	
	beta	p	beta	p
Cord:maternal IgG ratio	0.06	0.595	1.36	<0.001
Infant				
Male sex	0.07	0.067	0.00	0.850
Gestational age	0.04	0.006	0.01	0.549
Birthweight	0.08	0.102	-0.01	0.504
Maternal				
Age	0.01	0.001	0.00	0.246
Low education	-0.06	0.123	0.01	0.410
Gravidity	0.03	0.088	0.00	0.921
Log_{10} IgM	-0.14	0.006	-0.01	0.798
Log_{10} IgG	0.76	<0.001	1.01	<0.001
Season				
Jan-Mar	0.13	0.013	0.02	0.170
Apr-Jun	0.29	<0.001	0.08	<0.001
Jul-Sep	1.00	-	1.00	-
Oct-Dec	0.04	0.417	0.01	0.637

Beta values represent the slope of the linear association and p-values demonstrate whether the slope is significantly different from the null hypothesis of 0.

Figure 1: Anti-*S. sonnei*-O antibody levels in the first year of life in a cohort of 503 Vietnamese children

(A) Count of the number of assayed infant plasma samples at different ages in the first year after birth. Anti-*S. sonnei*-O IgG (B) and IgM (C) titers shown over time for each individual in the cohort on a $\log_{10}$ scale.

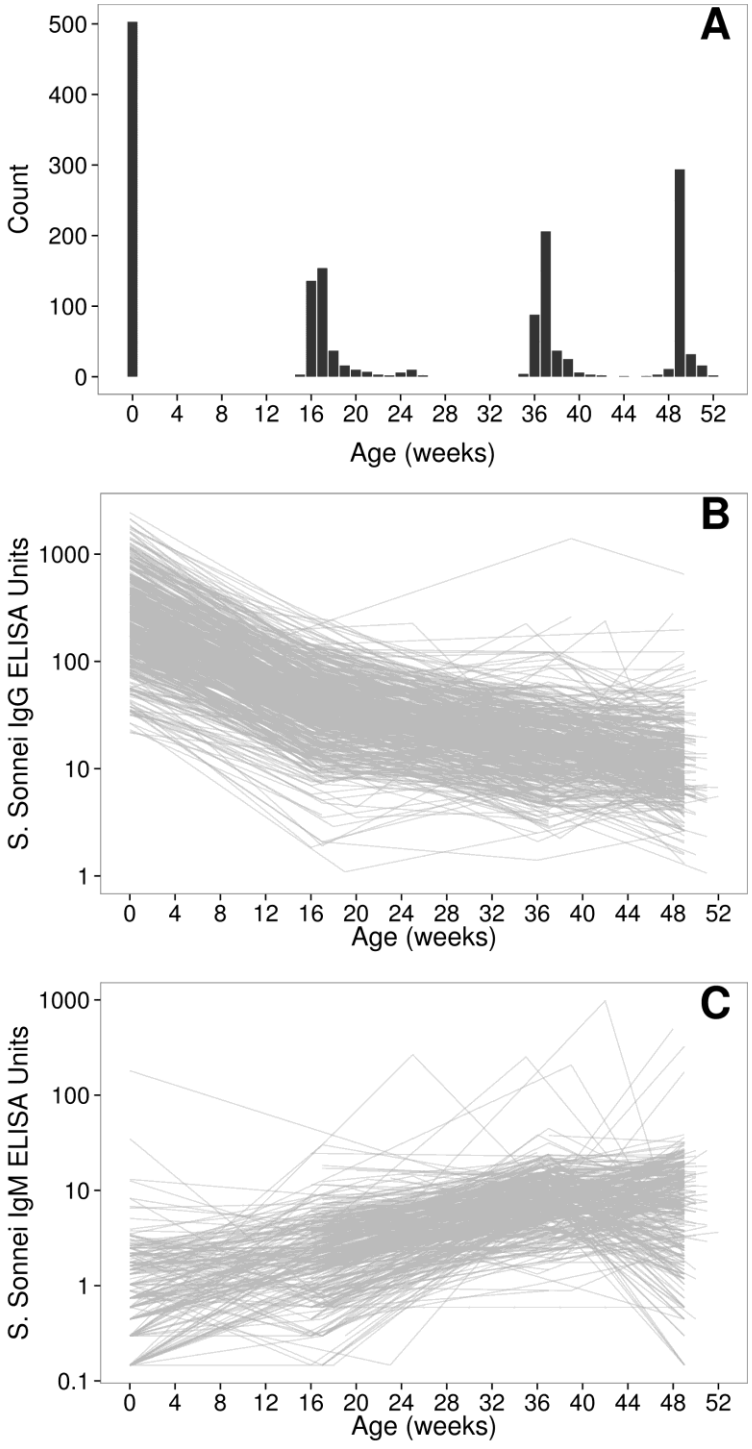


Figure 2: **Kaplan Meier curve showing the proportion of infants with detectable anti-*S. sonnei*-O IgG in the first year after birth**

The proportion of infants with detectable anti-*S. sonnei*-O IgG censored by (1) when their titer dropped below 10.3EU (see methods), (2) had any detectable increase in IgG titer or, (3) lost to follow up. The number of infants with detectable antibody at each time point are shown below the x-axis.

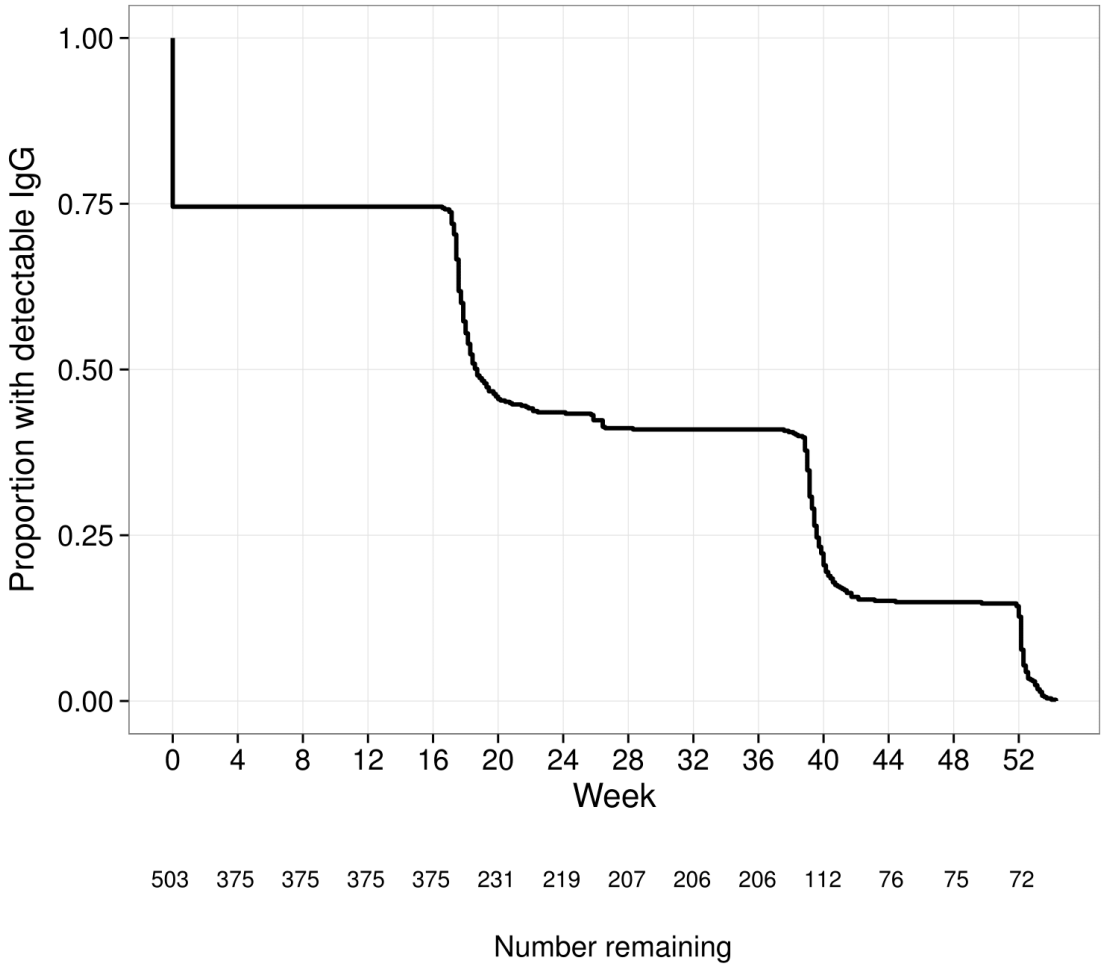
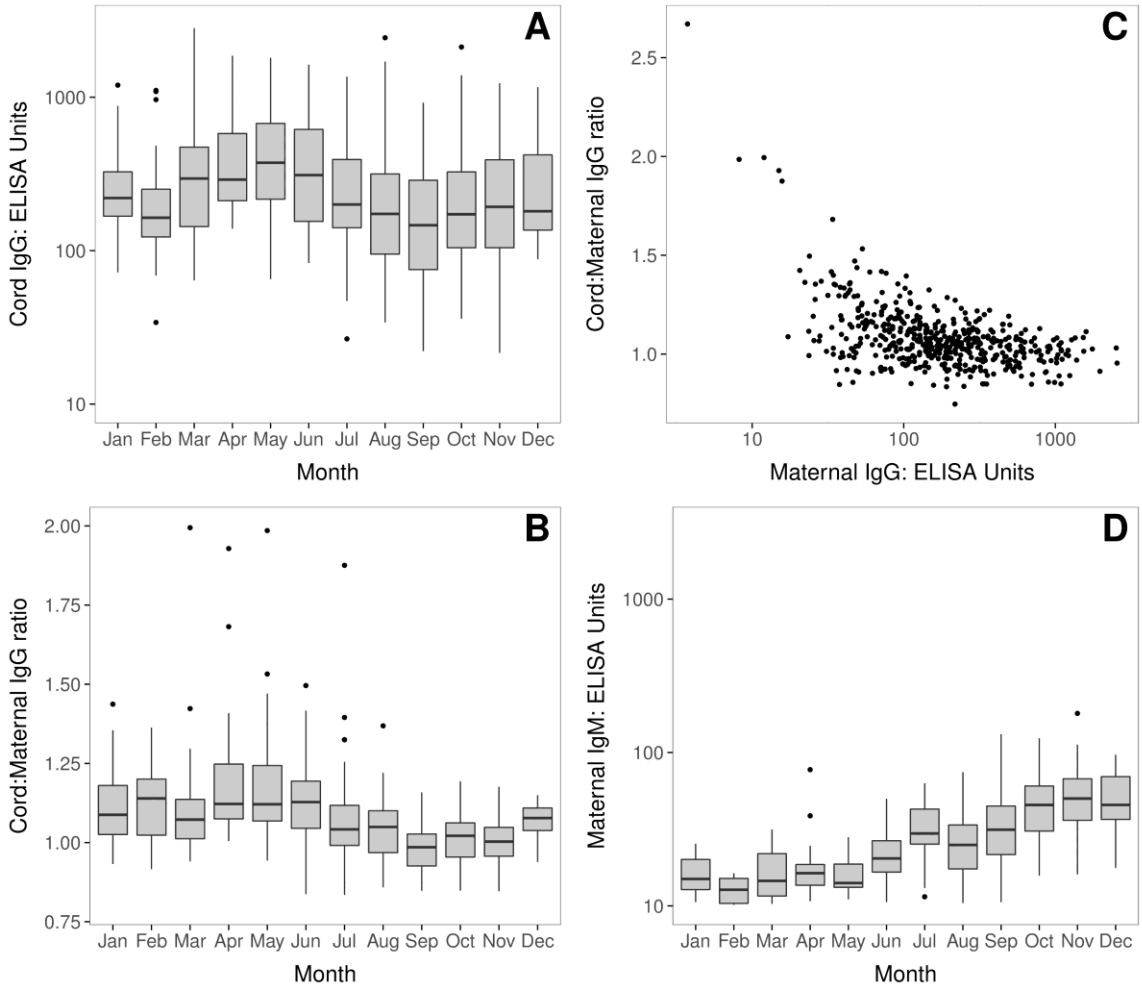


Figure 3: Temporal anti-*S. sonnei*-O antibody cord titers and transplacental transfer dynamics (A) Anti-*S. sonnei*-O IgG cord plasma titers shown by month of birth on a $\log_{10}$ scale. (B) The ratio of cord:matneral anti-*S. sonnei*-O IgG titer by month of birth. (C) Scatterplot showing the relationship between maternal anti-*S. sonnei*-O IgG titers and the ratio of cord:maternal plasma transfer. (D) Maternal anti-*S. sonnei*-O IgM titers shown by month of birth on a $\log_{10}$ scale.



10 RESEARCH PAPER 8: The clinical implications of reduced susceptibility to fluoroquinolones in paediatric *Shigella sonnei* and *Shigella flexneri* infections

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PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Corinne Thompson
Principal Supervisor	Stephen Baker
Thesis Title	The epidemiology of paediatric Shigella infection and disease in Ho Chi Minh City, Vietnam

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Journal of Antimicrobial Chemotherapy
Please list the paper's authors in the intended authorship order:	Corinne N Thompson, Nga Tran Vu Thieu, Phat Voong Vinh, Anh Nguyen Duc, Marcel Wolbers, Ha Vinh, James I Campbell, Dung Tran Thi Ngoc, Nguyen Van Minh Hoang, Tuyen Ha Thanh, Hao Chung The, To Nguyen Thi Nguyen, Nguyen Phu Huong Lan, Christopher M Parry, Nguyen Van Vinh Chau, Guy Thwaites, Duy Pham Thanh and Stephen Baker
Stage of publication	**In press**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am first author. I cleaned and analysed the primary data. I wrote the code for analysis in STATA and R with assistance from Dr Nguyen Duc Anh in the Biostatistics Department at OUCRU. I made all tables and figures for the work. I wrote the manuscript and was responsible for the submission.

Student Signature: _____ **Date:** 16NOV15

Supervisor Signature: _____ **Date:** 16/11/15

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Journal of Antimicrobial Chemotherapy

Clinical implications of reduced susceptibility to fluoroquinolones in paediatric *Shigella sonnei* and *Shigella flexneri* infections

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Objectives: We aimed to quantify the impact of fluoroquinolone resistance on the clinical outcome of paediatric shigellosis patients treated with fluoroquinolones in southern Vietnam. Such information is important to inform therapeutic management for infections caused by this increasingly drug-resistant pathogen, responsible for high morbidity and mortality in young children globally.

Methods: Clinical information and bacterial isolates were derived from a randomized controlled trial comparing gatifloxacin with ciprofloxacin for the treatment of paediatric shigellosis. Time–kill experiments were performed to evaluate the impact of MIC on the *in vitro* growth of *Shigella* and Cox regression modelling was used to compare clinical outcome between treatments and *Shigella* species.

Results: *Shigella flexneri* patients treated with gatifloxacin had significantly worse outcomes than those treated with ciprofloxacin. However, the MICs of fluoroquinolones were not significantly associated with poorer outcome. The presence of S83L and A87T mutations in the *gyrA* gene significantly increased MICs of fluoroquinolones. Finally, elevated MICs and the presence of the *qnrS* gene allowed *Shigella* to replicate efficiently *in vitro* in high concentrations of ciprofloxacin.

Conclusions: We found that below the CLSI breakpoint, there is no association between MIC and clinical outcome in paediatric shigellosis infections. However, *S. flexneri* patients had worse clinical outcomes when treated with gatifloxacin in this study regardless of MIC. Additionally, *Shigella* harbouring the *qnrS* gene are able to replicate efficiently in high concentrations of ciprofloxacin and we hypothesize that such strains possess a competitive advantage against fluoroquinolone-susceptible strains due to enhanced shedding and transmission.

Introduction

The Gram-negative bacterial genus *Shigella* are the most common cause of bacillary dysentery globally.[1,2] Of the four species within the genus, *Shigella flexneri* and *Shigella sonnei* predominate, with *S. sonnei* currently replacing *S. flexneri* as the major species in industrializing regions.[3] The WHO currently recommends the fluoroquinolone ciprofloxacin as the first-line therapy, with ceftriaxone and pivmecillinam as secondary alternatives.[4] However, antimicrobial resistance (AMR) within the species is becoming more prevalent and may present a significant challenge for therapeutic management.

The primary target of the fluoroquinolones is the DNA gyrase, a type II topoisomerase essential for DNA replication and transcription.[5] Mutations in the *gyrA* gene increase the MICs of fluoroquinolones for *Shigella* and other Enterobacteriaceae.[6–9] Plasmid-mediated quinolone resistance (PMQR) genes can also be acquired, such as the *qnr* genes that encode pentapeptide repeat proteins that bind to and protect the DNA gyrase and topoisomerase from the action of fluoroquinolones.[10] Complete ciprofloxacin resistance (MIC $\geq$4 mg/L[11]) has been recently reported in both domestic and imported *S. sonnei* isolates in the USA, Vietnam and elsewhere.[12–14]

The rapid evolution and global dissemination of fluoroquinolone resistance in the Enterobacteriaceae hampers effective

treatment and is, therefore, a major threat to human health.[15] The WHO has explicitly listed fluoroquinolone-resistant *Shigella* as one of its top concerns in the current international focus on AMR.[15] AMR can lead to inappropriate choice of antimicrobial for initial therapy and may force clinicians to choose more toxic or more expensive antimicrobials.[16] Furthermore, patients infected with fluoroquinolone-resistant *Campylobacter* and *Salmonella* infections in the USA have been shown to have a longer duration of diarrhoea compared with those infected with fluoroquinolone-susceptible strains.[17,18] Although one study from Vietnam suggested a correlation between increasing AMR levels in *S. sonnei* and clinical severity,[19] no rigorous evaluation of the impact of fluoroquinolone resistance or presence of *gyrA* mutations on clinical outcome of *Shigella* infections has been performed.

Here, we aimed to quantify the effect of fluoroquinolone choice, fluoroquinolone susceptibility and presence of *gyrA* mutations on fever clearance time (FCT) and total duration of illness in children with *S. flexneri* and *S. sonnei* infections in Vietnam. Additionally, we sought to compare the severity and AMR profiles between *Shigella* species as well as investigate the effect of elevated MIC and *gyrA* mutations on the *in vitro* activity of *Shigella*. Understanding the dynamics of increasing MICs of commonly used fluoroquinolones and clinical patient outcome in industrializing locations is important as it allows clinicians to be better informed when prescribing therapies for what can often be severe infections in young children.

Methods

Patient population

The source data for this study was a randomized controlled trial. The protocol (including justification for use of gatifloxacin) and results for this trial have been described previously in detail.[20] Briefly, 500 children were enrolled into an open-label, randomized clinical trial comparing 3 day regimens of gatifloxacin (10 mg/kg/day orally in one dose) and ciprofloxacin (30 mg/kg/day orally in two doses) for the treatment of shigellosis in southern Vietnam. Children were enrolled between 2006 and 2009 at the Hospital for Tropical Diseases in Ho Chi Minh City and at Huu Nghi Hospital in Dong Thap province. Inclusion criteria included age <15 years and a history of bloody or mucoid stools in the 72 h prior to admission to hospital. Exclusion criteria included severe infection (shock, jaundice and extensive gastrointestinal bleeding), known treatment with a fluoroquinolone during the episode and concomitant infection requiring antimicrobial therapy.

Study procedures

Daily case report forms detailing clinical presentation were administered for each patient during the period of hospitalization. A case report form was also administered at a follow-up visit that occurred 7 days after discharge. Clinical failure was defined as fever (≥37.8°C) or the persistence of any signs or symptoms after 120 h of start of treatment (vomiting, abdominal pain or tenesmus with/without three or more loose stools with/without blood and/or mucus). Total duration of symptoms was defined as the time from admission until cessation of all listed symptoms. Microbiological failure was defined as a positive stool culture for the original infecting pathogen after day 3 of the antimicrobial therapy. FCT was defined as the time from admission until temperature was ≤37.8°C for ≥48 h.

Stool samples were collected on admission and standard microbiological techniques were employed to identify *Shigella* and *Salmonella* isolates.[20] Antimicrobial susceptibility testing was performed by disc diffusion following methods prescribed by the CLSI.[11] MICs were calculated by Etest as per the manufacturer's instructions (AB Biodisk, Sweden). Strains that were identified as resistant to ceftriaxone were subjected to further phenotypic tests to confirm ESBL production using discs containing only cefotaxime (30 μg) and both cefotaxime and ceftazidime combined with clavulanic acid (10 μg), according to current CLSI guidelines.[11]

Genomic DNA extraction, PCR and sequencing

Genomic DNA was purified using the Wizard genomic DNA extraction kit (Promega, USA) as recommended by the manufacturer. Extracted DNA was subjected to PCR targeting known mutation regions on *gyrA* and *parC* genes and the PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrS*, *aac(6′)-Ib-cr* and *qepA*. Primer sequences were *GyrA*_F: 5′-CGACCTTGCGAGAGAAAT-3′, *GyrA*_R: 5′-GTTCCATCAGCCCTTCAA-3′,[21] *ParC*_F: 5′-AAACCTGTTCAGCGCC GCATT-3′ and *ParC*_R: 5′-GTGGTGCCGTTAAGCAAA-3′.[22] The primers for the PMQR genes were as previously published.[23–25] *Taq* DNA polymerase supplied by Bioline (UK) was used for the amplifications. Concentrations of reagents were as recommended by the manufacturers. PCR amplifications were performed under the following conditions: 1 cycle of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. PCR amplicons were sequenced using an ABI 3700 system (ABI, USA) and sequencing reactions were prepared as recommended by the manufacturer. Resulting sequences were analysed using Bioedit software.

Time–kill analyses

One isolate from each mutation group was selected for *in vitro* time–kill experiments, all were *S. flexneri* isolates: (i) no *gyrA* mutation, ciprofloxacin MIC 0.023 mg/L and gatifloxacin MIC 0.023 mg/L; (ii) *gyrA* mutation A87T, ciprofloxacin MIC 0.094 mg/L and gatifloxacin MIC 0.125 mg/L; (iii) *gyrA* mutation S83L, ciprofloxacin MIC 0.19 mg/L and gatifloxacin MIC 0.19 mg/L; and (iv) *gyrA* mutation S83L, ciprofloxacin MIC 8 mg/L and gatifloxacin MIC 6 mg/L with *qnrS* gene. Strains were grown overnight in Mueller–Hinton (MH) broth. *S. flexneri* isolates were chosen due to the larger range of MICs of fluoroquinolones compared with those for *S. sonnei*. The bacterial cultures were diluted 1:1000 into 10 mL of fresh MH broth. The inoculation was incubated at 37°C with a circular agitation speed of 150 rpm for 1.5 h. The cultures were mixed with either ciprofloxacin or gatifloxacin, which had been prepared in MH broth to achieve the final volume of 20 mL of the desired concentration of drugs. Controls for each mutant were identical cultures but without supplementary antimicrobials. Bacterial cells were counted at time 0 and 30 min, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h after incubation with antimicrobials. The experiment was performed in replicates of nine for each selected isolate. The limit of detection was 10 cfu/mL.[26]

Statistical analyses

All data were analysed in STATA v13 (TX, USA). Plots were made in R (R Foundation for Statistical Computing, Austria) using the ggplot2 package.[27] Continuous data were compared between groups using the Kruskal–Wallis test. Categorical group data were compared using χ^2 or Fisher's exact test. For MICs that were recorded as 'greater than X' or 'less than X', these values were converted into 2X and X/2, respectively. Logistic regression was used to evaluate the relationship between treatment arm, MICs and overall failure, with interaction between drug and MIC evaluated through the likelihood ratio test. We analysed the time to event endpoints of FCT and total duration of symptoms using Cox regression models. Interaction between species and treatment arm as well as MIC and treatment within species were evaluated using the likelihood ratio test. Age was included *a priori* as a covariate in all models.

Ethics approval

This study was approved by the institutional ethics review boards of the Hospital for Tropical Diseases and Huu Nghi Hospital and the Oxford Tropical Research Ethics Committee (OxTREC: 010-06). Written informed consent from the parent or guardian was required for enrolment into the trial.

Results

Baseline clinical and demographic characteristics

Of the 500 children enrolled, 6 withdrew after randomization, leaving 494 for analysis. A total of 107/494 (22%) enrolled trial patients were stool culture positive for *Shigella* spp. Of these, 72 (67%) were *S. sonnei*, 33 (31%) were *S. flexneri* and 2 (2%) were *Shigella boydii*. As shown in Table 1, *S. sonnei* patients were slightly younger (median: 30 months, IQR: 20–43) than *S. flexneri* patients (median: 36 months, IQR: 22–60) and were more likely to report a greater number of mucoid stools in the first 24 h after admission compared with *S. flexneri* patients. *S. sonnei* patients also had slightly elevated white cell counts (median 13.5×10^9/L, IQR: 10.7–16.9) compared with *S. flexneri* patients (median: 10.2×10^9/L, IQR: 7.3–16.4). *S. flexneri* patients, however, were more likely to report abdominal pain (91%) prior to admission than *S. sonnei* patients (72%).

Table 1. Baseline demographic and clinical characteristics of all patients, *S. sonnei* patients and *S. flexneri* patients

Characteristic	All patients, $n=494$	*S. sonnei* patients, $n=72$	*S. flexneri* patients, $n=33$	P^a
Site, *n* (%)				
Ho Chi Minh City	194 (39.3)	48 (66.7)	20 (60.6)	0.546
Dong Thap	300 (60.7)	24 (33.3)	13 (39.4)	
Study drug, *n* (%)				
ciprofloxacin	245 (49.6)	34 (47.2)	12 (36.4)	0.298
gatifloxacin	248 (50.2)	38 (52.8)	21 (63.6)	
Age (months), median (IQR)	19 (10.5–32)	30 (20–43)	36 (22–60)	0.062
Male, *n* (%)	291 (58.9)	40 (55.6)	14 (42.4)	0.211
Nutritional status, *n* (%)				
overweight	4 (0.8)	1 (1.4)	0 (0)	0.089
normal	363 (73.5)	60 (83.3)	26 (78.8)	
malnutrition I	93 (18.8)	10 (13.9)	3 (9.1)	
malnutrition II	29 (5.9)	1 (1.4)	4 (12.1)	
malnutrition III	5 (1.0)	0 (0)	0 (0)	
Prior to admission				
illness duration (h), median (IQR)	24 (16–48)	20 (12–33)	19 (12–24)	0.785
fever (≥37.8°C), *n* (%)	429 (87.4)	68 (94.4)	33 (100)	0.167
history of febrile convulsions, *n* (%)	40 (8.1)	6 (8.3)	2 (6.1)	1.000
history of diarrhoea with blood, *n* (%)	210 (42.5)	24 (33.3)	11 (33.3)	1.000
history of mucoid diarrhoea without blood, *n* (%)	284 (57.5)	48 (66.7)	22 (66.7)	1.000
vomiting, *n* (%)	204 (41.3)	34 (47.2)	16 (48.5)	0.904
abdominal pain, *n* (%)	365/492 (74.2)	52 (72.2)	30 (90.9)	0.041
tenesmus, *n* (%)	339/490 (69.2)	45/71 (63.4)	22 (66.7)	0.745
Within 24 h of admission				
mucoid diarrhoea without blood, *n* (%)	370 (74.9)	59 (81.9)	22 (66.7)	0.083
number of mucoid stools/24 h, median (IQR)	3 (0–6)	4 (2–10)	2 (0–5)	0.028
diarrhoea with blood, *n* (%)	445 (90.1)	70 (97.2)	33 (100)	0.334
number of bloody stools/24 h, median (IQR)	1 (1–5)	3 (1–6)	3 (1–9)	0.187
maximum number of episodes/24 h, median (IQR)	6 (3–10)	8 (3–11)	8 (4–10)	0.806
white blood cells in stool (cells/HPF), *n* (%)				
0	214/479 (44.7)	14/68 (20.6)	8/32 (25.0)	0.754
1–10	79/479 (16.5)	11/68 (16.2)	4/32 (12.5)	
11–20	42/479 (8.8)	5/68 (7.4)	4/32 (12.5)	
21–30	104/479 (21.7)	26/68 (38.2)	9/32 (28.1)	
>30	40/479 (8.4)	12/68 (17.6)	7/32 (21.9)	
white cell count $\times10^9$/L, median (IQR)	11.3 (8–14.9)	13.5 (10.7–16.9)	10.2 (7.3–16.4)	0.071

HPF, high power field.
[a]*P* value comparing *S. sonnei* and *S. flexneri* by Kruskal–Wallis test for continuous data or χ^2/Fisher's exact test for categorical data.

Table 2. Comparison of MICs of fluoroquinolones for *S. sonnei* and *S. flexneri* isolates

Antimicrobial	*S. sonnei* MIC (mg/L), $n=72$	*S. flexneri* MIC (mg/L), $n=33$	P^a
Nalidixic acid			
median (range)	48 (0.09–512)	512 (1–512)	0.046
geometric mean	35.3	68.4	
Ciprofloxacin			
median (range)	0.064 (0.01–0.25)	0.125 (0.01–8)	0.011
geometric mean	0.05	0.08	
Gatifloxacin			
median (range)	0.094 (0.01–0.25)	0.19 (0.01–6)	0.008
geometric mean	0.06	0.09	
Ofloxacin			
median (range)	0.38 (0.05–1)	0.38 (0.05–16)	0.135
geometric mean	0.25	0.3	

[a]*P* value comparing MIC between species by Kruskal–Wallis test.

Antimicrobial susceptibility

As shown in Table 2, *S. flexneri* isolates were more likely to have higher MICs against the fluoroquinolones (with the exception of ofloxacin) compared to *S. sonnei*. In fact, *S. flexneri* had significantly higher MICs of all tested antimicrobials with the exception of ceftriaxone, for which *S. sonnei* had significantly elevated MICs over *S. flexneri* ($P<0.001$, Kruskal–Wallis test) (Figure 1). Overall, *S. flexneri* isolates were more likely to be MDR (defined here as non-susceptible to more than three antimicrobial classes) (28/33, 85%) compared with *S. sonnei* (22/72, 31%) ($P<0.001$, Fisher's exact test) while *S. sonnei* isolates were more likely to exhibit an ESBL phenotype (14/72, 19%) than *S. flexneri* (1/33, 3%) ($P=0.033$, Fisher's exact test) (Figure S1, available as Supplementary data at *JAC* Online). Finally, 3/33 (9%) of the tested *S. flexneri* isolates and 1/72 (1%) of tested *S. sonnei* isolates were PCR amplification positive for the *qnrS* gene. No *parC* mutations, *qnrA*, *qnrB*, *aac(6′)-Ib-cr* or *qepA* genes were identified within any of the *Shigella* isolates.

The MICs of fluoroquinolones were highly correlated for both *S. sonnei* and *S. flexneri* ($P<0.001$ for all correlations). When normalized to the mean of the current CLSI resistance breakpoint (ciprofloxacin: ≥4 mg/L, gatifloxacin: ≥8 mg/L),[11] both *S. flexneri* and *S. sonnei* had higher relative median $\log_2$ MICs of ciprofloxacin

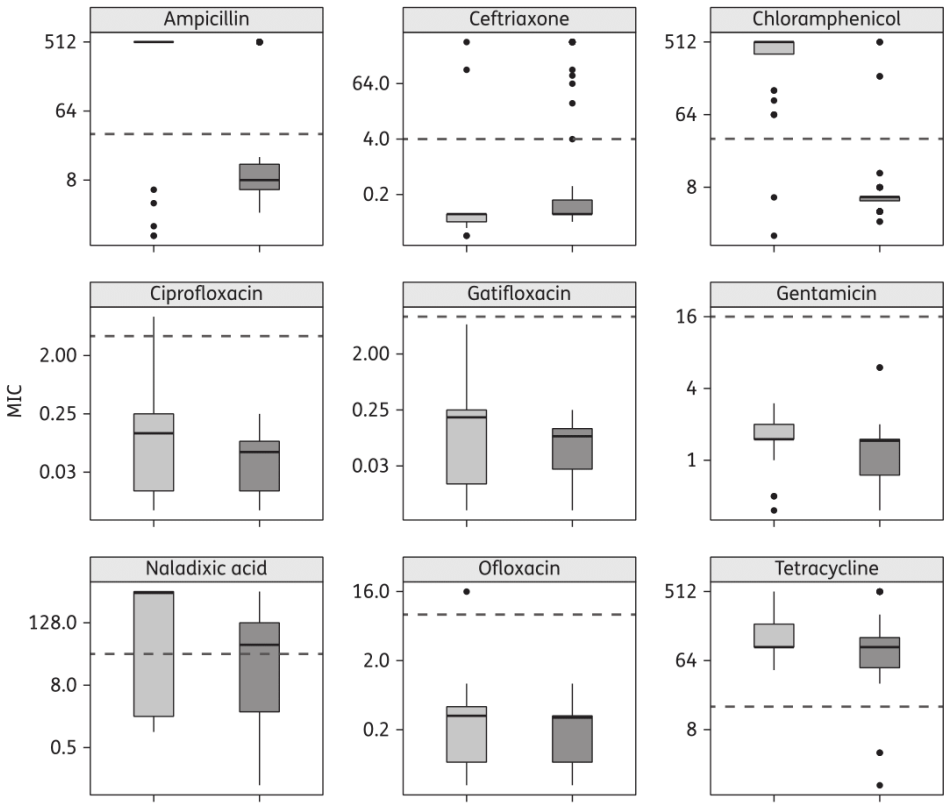

Figure 1. MICs (mg/L) of a range of antimicrobials for the *Shigella* isolates in this study ($\log_2$ scale). Box plots show the median (black line across each box) and the 5th and 95th percentiles. MICs for *S. sonnei* are shown in dark grey and MICs for *S. flexneri* are in light grey. The broken line in each plot represents the current CLSI breakpoint for resistance.[11]

than of gatifloxacin ($P<0.001$ in both cases, Kruskal–Wallis test of *z*-score) (Figure S2). Furthermore, the presence of a single *gyrA* mutation and/or the *qnrS* gene dramatically increased the MICs of fluoroquinolones for both *S. sonnei* and *S. flexneri* (Table 3 and Figure S3).

Time–kill curves

Mutations in *gyrA*, the presence of *qnrS* and elevated MICs of ciprofloxacin substantially increased the ability of *S. flexneri* to replicate in the presence of ciprofloxacin (Figure 2). The *S. flexneri* strain lacking a *gyrA* mutation with a low MIC of ciprofloxacin was rapidly killed by the antimicrobial (Figure 2). While the isolate with the *gyrA* mutation A87T (Figure 2) was still effectively killed at higher concentrations of ciprofloxacin, bacterial growth remained elevated at lower concentrations compared with the *S. flexneri* without a *gyrA* mutation. The S83L mutation (Figure 2) reduced the bactericidal activity of ciprofloxacin even further, with cfu/mL reduced by >5 logs at high concentrations. Finally, the *S. flexneri* isolate with an S83L *gyrA* mutation, *qnrS* gene and MIC of ciprofloxacin of 8 mg/L (Figure 2) was able to grow in the presence of all tested concentrations of ciprofloxacin during the first hour post-inoculation, with growth falling by 2 logs only within the second hour post-inoculation. Further, the concentration-dependent killing effect was lost with the *qnrS* mutant, as the 2× to 16× MIC curves exhibited almost identical profiles. The same pattern was observed with the corresponding gatifloxacin time–kill curve (data not shown).

Clinical outcome in shigellosis with fluoroquinolone therapy

Overall, there were 8 (7.6%) failures among the 105 *Shigella* cases, all of which were *S. sonnei* (8/72, 11%). Two of these cases were microbiological failures and six were classified as clinical failures. Overall failure amongst the *S. sonnei* isolates was not significantly associated with either treatment drug (OR: 0.5, 95% CI: 0.1–2.3, $P=0.37$) or MIC of nalidixic acid, ciprofloxacin, gatifloxacin or ofloxacin. Additionally, although 5/8 (63%) failures had the A87T *gyrA* mutation and 1/8 (13%) had the S83L *gyrA* mutation, there was no significant effect of a *gyrA* mutation on failure (OR: 1.03, 95% CI: 0.3–3.3, $P=0.96$).

Overall, the FCTs of *S. sonnei* and *S. flexneri* were not significantly different (HR: 1.2, 95% CI: 0.8–1.8, $P=0.47$). There was no difference in FCT between the two species in the ciprofloxacin arm (HR: 0.73, 95% CI: 0.4–1.4, $P=0.355$) and although *S. flexneri* patients had moderately longer FCTs in the gatifloxacin arm (median: 27 h, IQR: 16–48) compared with *S. sonnei* (median: 12 h, IQR: 3–24), the difference was not statistically significant after controlling for age (HR: 1.52, 95% CI: 0.9–2.7, $P=0.13$) (Figure 3a and b). Notably, the FCTs of *S. flexneri* treated with gatifloxacin (median: 27 h, IQR: 16–48) were significantly longer than for *S. flexneri* patients treated with ciprofloxacin (median: 15, IQR: 4–22) (HR: 0.40, 95% CI: 0.2–0.9, $P=0.02$). Similar patterns were observed for total duration of symptoms (Figure 3c and d), as *S. flexneri* patients treated with gatifloxacin (median: 60 h, IQR: 4–72) had significantly longer duration of symptoms compared with *S. flexneri* patients treated with ciprofloxacin (median: 48 h, IQR: 33–54) (HR: 0.39, 95% CI: 0.2–0.9, $P=0.02$). No difference in total duration of symptoms was detected between the treatment arms for *S. sonnei* patients. The presence of *gyrA* mutations did not have a significant effect on either FCT or total duration of symptoms ($P>0.05$ for both comparisons).

When evaluating the effect of MICs on FCT and duration of symptoms, we found a significant interaction between treatment arm and gatifloxacin and ciprofloxacin MICs for *S. flexneri* patients ($P<0.03$ in all comparisons). Although increasing ciprofloxacin MICs appeared to be associated with increased FCT and symptom duration in *S. flexneri* patients treated with gatifloxacin (Figure 4), these trends were not statistically significant (effect of $\log_2$ ciprofloxacin MIC on (i) FCT: HR: 0.92 (95% CI: 0.8–1.1, $P=0.33$); and (ii) symptom duration: HR: 0.86 (95% CI: 0.7–1.1, $P=0.15$). This pattern was comparable for the gatifloxacin MIC (data not shown). Finally, there was no significant effect of MIC of either antimicrobial in the ciprofloxacin treatment arm or amongst *S. sonnei* patients in either treatment arm.

Discussion

The bacterial genus *Shigella* are a considerable public health problem, responsible for >7 million disability-adjusted life years and >100 000 deaths annually, which are mainly concentrated in children living in developing countries.[1,2,28,29] The current drug of choice for treating *Shigella* infections is ciprofloxacin. However, fluoroquinolone resistance is threatening to make the

Table 3. MICs of fluoroquinolones for *S. sonnei* and *S. flexneri* by *gyrA* mutation and *qnrS* gene, median (range)

	S. sonnei MIC (mg/L)				*S. flexneri* MIC (mg/L)			
Antimicrobial	no mutation, $n=19$	A87T, $n=40$	S83L, $n=9$	S83L/*qnrS*, $n=1$	no mutation, $n=11$	S83L, $n=16$	*qnrS*, $n=1$	S83L/*qnrS*, $n=2$
Nalidixic acid	1.50 (0.09–3.0)	64 (32–512)	96 (64–512)	512	2.0 (1.0–4.0)	512 (512–512)	1.50	512 (512–512)
Ciprofloxacin	0.01 (0.01–0.02)	0.06 (0.05–0.13)	0.19 (0.13–0.25)	0.25	0.02 (0.01–0.05)	0.19 (0.09–0.25)	0.01	4.13 (0.25–8.0)
Gatifloxacin	0.01 (0.01–0.13)	0.09 (0.06–0.13)	0.19 (0.13–0.25)	0.19	0.01 (0.01–0.06)	0.19 (0.13–0.38)	0.01	3.13 (0.25–6.0)
Ofloxacin	0.06 (0.05–0.09)	0.38 (0.25–0.50)	0.75 (0.5–1.0)	0.75	0.09 (0.05–0.19)	0.5 (0.38–1.0)	0.05	8.25 (0.5–16)

All pairwise comparisons within species across antimicrobials were statistically significantly different by Kruskal–Wallis tests (all $P<0.001$) and all pairwise comparisons of A87T and S83L (regardless of *qnrS*) in *S. sonnei* were statistically significantly different ($P<0.001$), except for A87T versus S83L for nalidixic acid ($P=0.03$), by Kruskal–Wallis test.

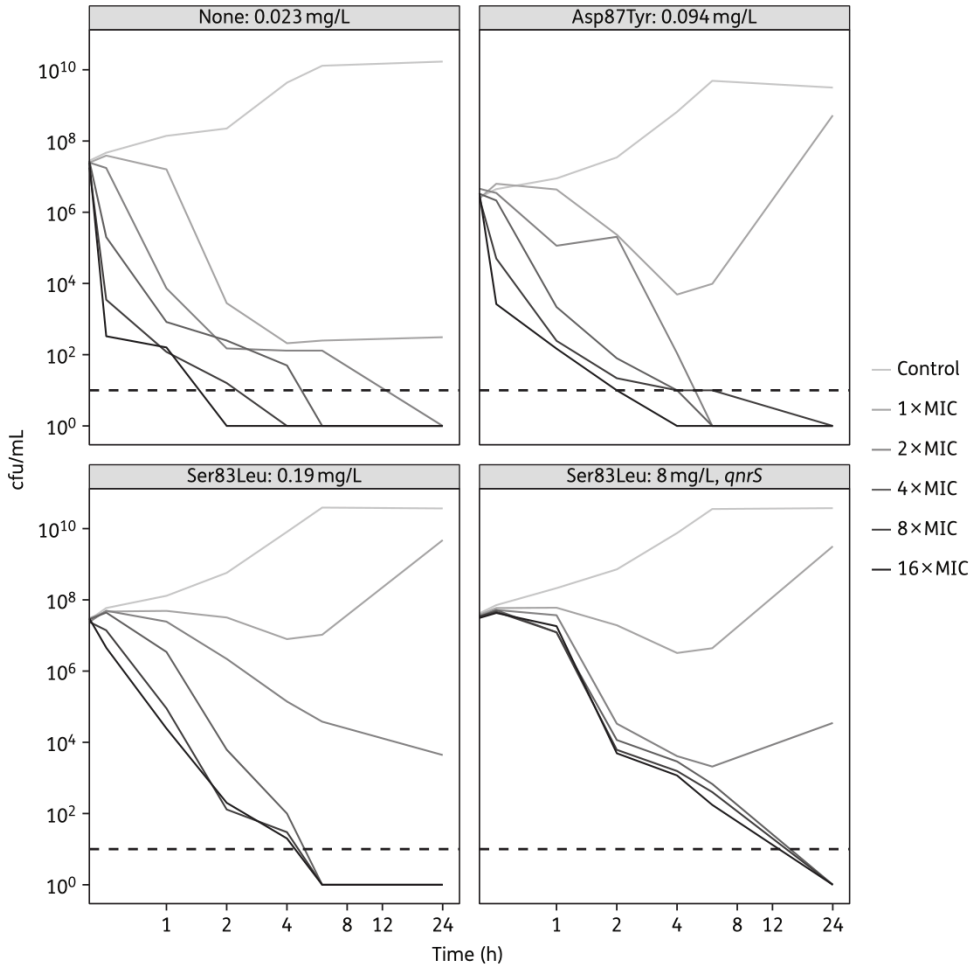

Figure 2. Time–kill curves of *S. flexneri gyrA* (*qnrS*) genotypes on exposure to increasing concentrations of ciprofloxacin. Plots showing the mean time–kills of *S. flexneri* isolates grown with increasing concentrations of ciprofloxacin based on the MIC for the original isolate at different timepoints post-inoculation ($\log_2$ scale). The *S. flexneri* isolates are: (a) no *gyrA* mutation, ciprofloxacin MIC = 0.023 mg/L; (b) *gyrA* mutation A87T, ciprofloxacin MIC = 0.094 mg/L; (c) *gyrA* mutation S83L, ciprofloxacin MIC = 0.19 mg/L; and (d) *gyrA* mutation S83L, ciprofloxacin MIC = 8 mg/L with the *qnrS* gene. The broken line represents the limit of detection for the assay (10 cfu/mL).

management of this diarrhoeal pathogen even more challenging.[15] Here, we aimed to quantify the impact of treatment choice, fluoroquinolone MIC and presence of *gyrA* mutations on clinical outcome of paediatric *S. flexneri* and *S. sonnei* patients treated with fluoroquinolones. We additionally evaluated the ability of *Shigella* strains of varying resistance profiles to grow in the presence of ciprofloxacin through time–kill experiments rather than single timepoint MIC testing.

While we hypothesized that poorer clinical outcome was associated with higher fluoroquinolone MIC and observed this trend in our data, we were unable to conclude that such relationships were statistically significant. This is likely due to small numbers of patients and relatively low MICs for isolates collected during the time period (2006–09). Mutations in *gyrA* were not found to be associated with an inferior clinical outcome in either species, although S83L and A87T mutations did result in isolates with higher MICs of fluoroquinolones. We hypothesize that fluoroquinolone MICs may play a greater role in clinical outcome above the CLSI breakpoint as we have shown conclusively that below the current breakpoints, MIC does not have a significant impact on patient outcome. Therefore, we surmise that the current CLSI breakpoints for fluoroquinolones are appropriate for *Shigella* therapy.

We identified differences in clinical response between both the *Shigella* species and treatment arms. *S. flexneri* patients had significantly longer FCTs and duration of symptoms when treated with gatifloxacin compared with ciprofloxacin. Gatifloxacin is presumed to be a more efficacious drug than ciprofloxacin,[30] yet *S. flexneri* isolates were more likely to have higher MICs of ciprofloxacin (relative to the CLSI breakpoint) compared with gatifloxacin. In the absence of additional data assessing the penetration of gatifloxacin into the epithelial cells lining the gastrointestinal tract, we hypothesize this difference between antimicrobials in

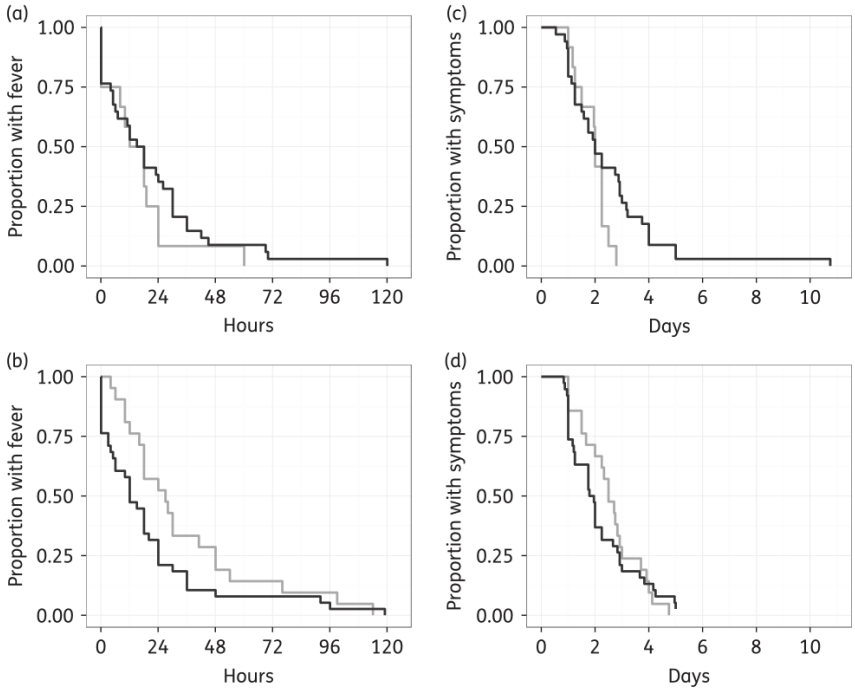

Figure 3. Clinical outcome comparison between *S. sonnei* and *S. flexneri* infections treated with fluoroquinolones. Unadjusted Kaplan–Meier plots showing FCT in hours in patients treated with (a) ciprofloxacin ($n=46$) and (b) gatifloxacin ($n=59$). Total duration of symptoms in days is shown in patients treated with (c) ciprofloxacin and (d) gatifloxacin. *S. sonnei* are shown in dark grey and *S. flexneri* are shown in light grey.

S. flexneri patients may be due to more effective killing of the local commensal gut microbiota by gatifloxacin, which may allow *Shigella* to more efficiently invade the gut tissue.[31–33] Further investigation into the effects of fluoroquinolones on the gut microbiota are required.

The time–kill experiments clearly demonstrate that an elevated MIC, and most notably presence of the combination of a *qnrS* gene and a *gyrA* mutation, allows *Shigella* to replicate efficiently in high concentrations of fluoroquinolone. We hypothesize that such enhanced persistence in the gastrointestinal tract during therapy endows fluoroquinolone-resistant *Shigella* with a competitive advantage over fluoroquinolone-susceptible strains. AMR *Shigella* are not only more likely to survive therapy but also to be shed and further transmitted in the community, over time replacing the fluoroquinolone-susceptible strains. For example, the acquisition of chloramphenicol resistance is understood to have afforded certain clones of *Salmonella enterica* serovar Typhimurium the ability to replace susceptible strains in sub-Saharan Africa over several years.[34] Furthermore, there is limited evidence that suggests that an elevated MIC of nalidixic acid lengthens the duration of excretion of *Shigella*.[35]

The time–kill results also suggest that acquisition of *qnrS* in addition to an S83L mutation in *gyrA* may lead to a loss of concentration-dependent killing by fluoroquinolones. This has been observed previously in *Escherichia coli* with the *qnrS* gene[36] and may relate to the mechanism of action of *qnrS* whereby peptides bind to the DNA gyrase and block fluoroquinolone activity. Although higher concentrations of fluoroquinolones are generally preferred in therapy to reduce the likelihood of development of resistant strains,[37,38] *qnrS* mutants may not respond to high doses given our time–kill observations. Therefore, combination therapy in patients with *qnrS*-positive *Shigella* infections could be warranted. Further pharmacodynamic work on *qnrS* mutants in the Enterobacteriaceae is clearly required.

This study has some limitations. First, FCT and duration of symptoms may not be the most appropriate measures of clinical outcome. We therefore may have under- or overestimated the effect of fluoroquinolone resistance on patient outcome. Second, we had a limited range of fluoroquinolone MICs and a limited number of treatment failures, which made it difficult to better evaluate trends in the isolates with elevated MICs or in those that failed treatment. It will be important to repeat this work with contemporary strains as higher levels of fluoroquinolone resistance will permit such an investigation.[39] Furthermore, collection of longitudinal stool samples after treatment is warranted to understand the effect of resistance on excretion duration. Nonetheless, the work presented here offers the first rigorous analysis of fluoroquinolone resistance on patient outcome to our knowledge and fills an important gap in the knowledge of this increasingly antimicrobial-resistant pathogen.

We conclude that below the CLSI breakpoint, MICs of fluoroquinolones do not strongly impact patient outcome in shigellosis. Therefore, the current CLSI breakpoints are warranted for *Shigella* infections. However, our data suggest that the choice of

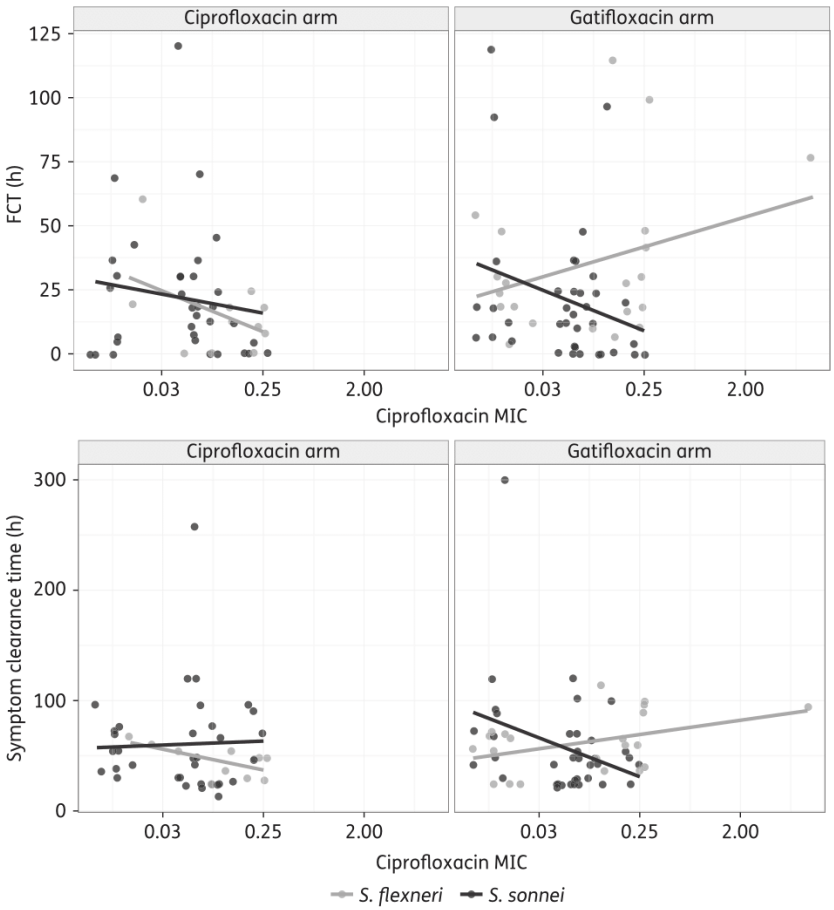

Figure 4. Effect of MIC of ciprofloxacin (mg/L) on clinical outcome for *S. sonnei* and *S. flexneri* infections. Associations between ciprofloxacin and FCT (top two plots) and symptom clearance time (bottom two plots) are shown. Patients treated with ciprofloxacin are shown on the left and patients treated with gatifloxacin are on the right. Patients infected with *S. sonnei* are shown in dark grey and patients infected with *S. flexneri* are in light grey. The lines represent the best-fit linear model for each set of patients.

fluoroquinolone is important in the management of *Shigella* infections, as *S. flexneri* patients treated with gatifloxacin had poorer outcomes compared with those treated with ciprofloxacin. Further, we demonstrate that *qnrS*-harbouring *Shigella* are able to grow effectively *in vitro* at high concentrations of ciprofloxacin and hypothesize that fluoroquinolone-resistant strains outcompete susceptible strains, as they are maintained during therapy, shed and therefore more likely to be transmitted in the community. Continued evaluation of the impact of fluoroquinolone resistance on the clinical outcome of *Shigella* patients over time is critical to help inform clinical treatment decisions for diarrhoeal infections.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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10.1 Supplementary figures

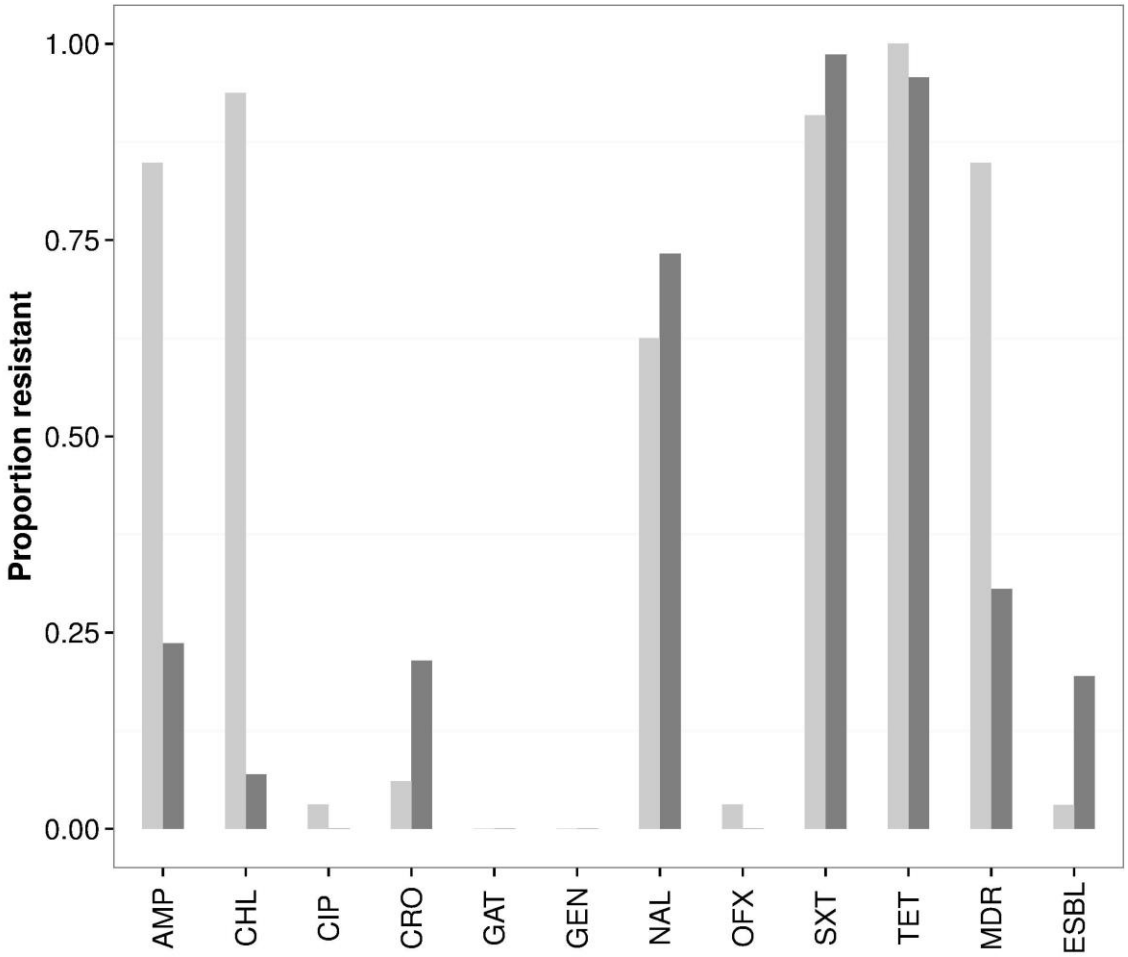

Figure S1: **The proportions of *S. flexneri* and *S. sonnei* isolates exhibiting antimicrobial resistance**

A bar chart showing the proportion of isolates fully resistant to each antimicrobial according to 2014 CLSI guidelines.[5] *S. sonnei* are shown in dark grey and *S. flexneri* in light grey. AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; CRO: ceftriaxone; GAT: gatifloxacin; GEN: gentamycin; NAL: nalidixic acid; OFX: ofloxacin; SXT: cotrimoxazole; TET: tetracycline; MDR: multidrug resistant (defined as resistance to ampicillin, chloramphenicol and cotrimoxazole); ESBL: extended-spectrum beta lactamase phenotype.

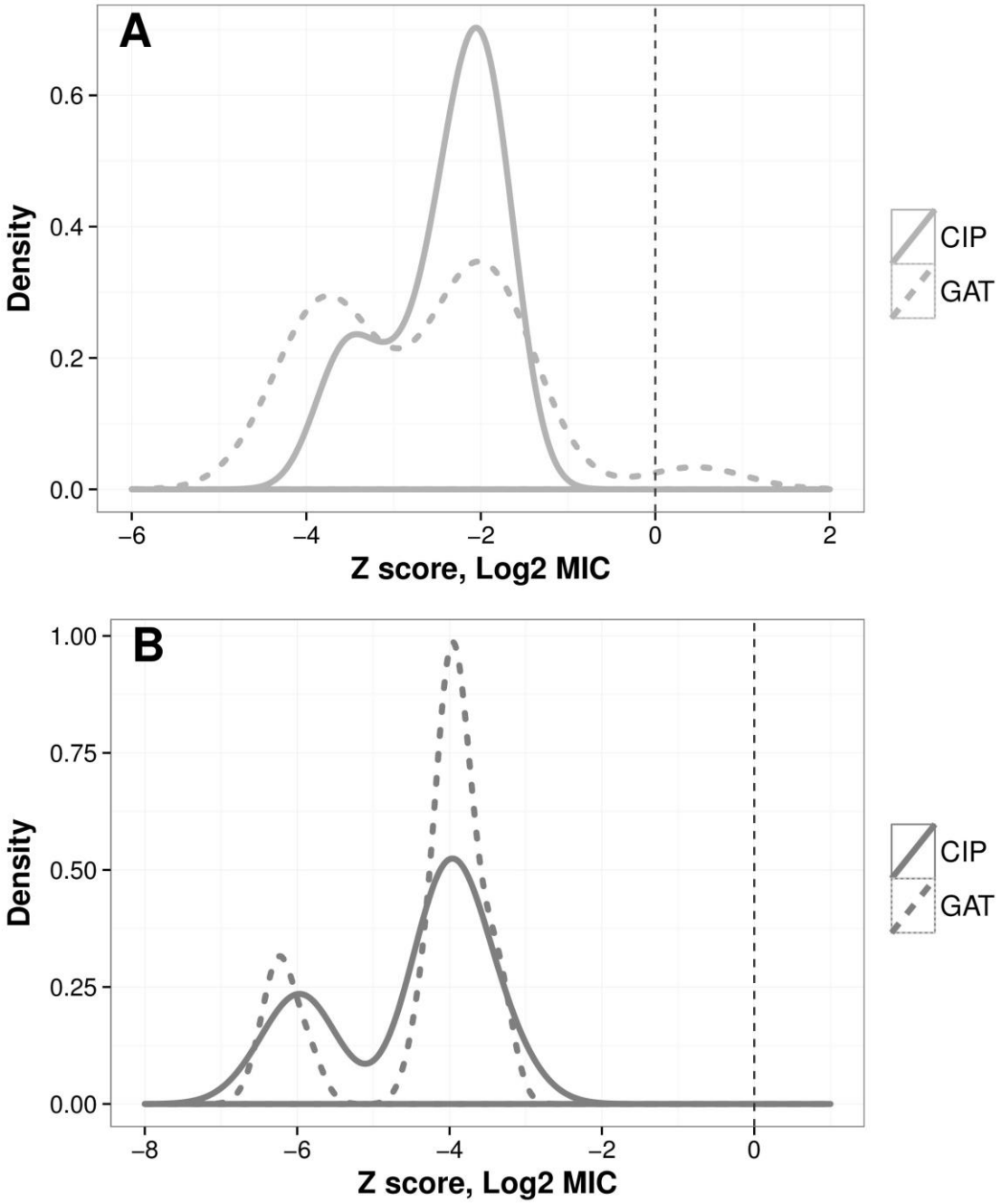

Figure S2: **Distribution of minimum inhibitory concentrations of *S. sonnei* and *S. flexneri* against ciprofloxacin and gatifloxacin relative to the CLSI resistance breakpoint** Density plots showing the Z-scores of log2MICs (mg/L) with the mean cantered on the CLSI breakpoint (ciprofloxacin: 1mg/L, gatifloxacin: 8mg/L). Gatifloxacin MICs are shown in the dashed line and ciprofloxacin MICs are shown in the solid line. Plots are separated by species: (A) *S. flexneri* and (B) *S. sonnei*. CIP: ciprofloxacin; GAT: gatifloxacin.

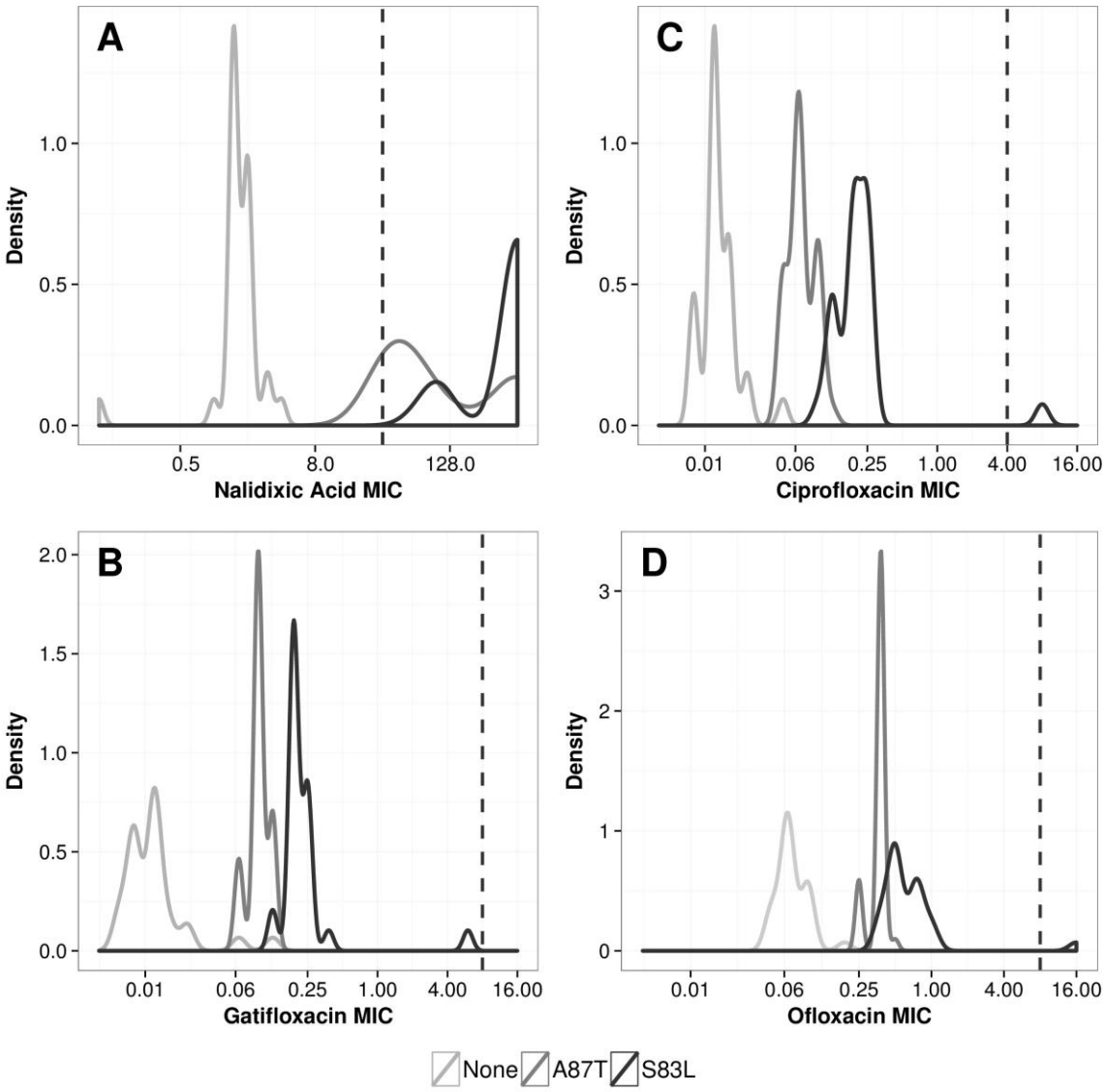

Figure S3: **The distribution of minimum inhibitory concentrations of *gyrA* mutations against four fluoroquinolones**

Density plots showing MICs (mg/L) to the (fluoro)quinolones for each *gyrA* mutation. Isolates without a *gyrA* mutation are shown in light grey, isolates with the A87T mutation are shown in dark grey and isolates with the S83L mutation are shown in black. The MICs of (A) nalidixic acid (B) gatifloxacin (C) ciprofloxacin and (D) ofloxacin are shown on a log2 scale.

11 DISCUSSION

Diarrhoeal disease continues to plague children living in disadvantaged societies. Diarrhoea (at least three loose or watery stools in a 24 hour period [1]) not only presents an often significant mortality risk in early life, but repeat infections during childhood lead to reduced physical and cognitive development [2–6]. The MDGs have witnessed a huge drop in childhood mortality globally since 1990 [7], but children in poor regions are disproportionately more likely to die or suffer from morbidity due to diarrhoea [8]. Diarrhoeal infections due to the Gram-negative bacteria *Shigella* are of particular concern due to the clinical severity, ease of spread and alarming levels of AMR [9–11]. Promising *Shigella* vaccine candidates are under development [12]. Vietnam, though progressing economically [13], still faces a considerable burden of childhood morbidity due to diarrhoeal disease [14]. In order for an effective *Shigella* vaccine to be potentially be introduced as a public health tool in this setting, more information regarding the epidemiology of the pathogen is required. Therefore, the principal aim of this thesis was to quantify the burden of diarrhoeal disease in HCMC, with a specific focus on *Shigella* infections, to determine whether a *Shigella* vaccine is necessary in this setting.

11.1 Summary of key findings

Work from this thesis has confirmed that diarrhoeal disease remains a significant cause of childhood morbidity in HCMC, with a community-based incidence of 70 episodes/100 child years of observation (CYO). Furthermore, *Shigella* incidence was found to be 1.5/100 CYO in children aged 2-5 years in the community which is substantial, but not as high as rotavirus (4.8/100 CYO) or norovirus (12.5/100 CYO). Children living at low elevation in the centre of the city were found to be at increased risk of hospitalised diarrhoeal disease, particularly during periods of elevated temperature of river levels. Additionally, AMR was identified against a variety of antimicrobials in *Shigella*, and organisms harbouring mutations against fluoroquinolone activity were shown to survive for a longer duration in the presence of ciprofloxacin *in vitro*, suggesting an epidemiological advantage of these strains in the face of growing fluoroquinolone resistance. Finally, maternal antibodies against *S. sonnei* are efficiently transferred across the placenta during pregnancy and circulate in the infant for 43 days in HCMC, which should aid in determination of an eventual vaccination schedule in locations where it is necessary.

In conclusion, data presented in this thesis do not warrant a *Shigella* vaccine presently in HCMC as the burden is not large relative to viral infections such as rotavirus and

norovirus. However, given the relentless growth of AMR in *Shigella* in addition to recent reports of Stx-producing *S. flexneri* and *S. sonnei* [15,16], vigilant epidemiological and molecular surveillance is required. Should either fully resistant (to first and second line therapies) or Stx-producing *Shigella* come to predominate in HCMC in the future, a vaccine may well prove necessary and economically feasible to avert a likely increase in morbidity and mortality. In the meantime, promotion of an integrated set of preventive interventions such as breastfeeding and water, sanitation and hygiene (WASH) measures is warranted.

11.2 Aetiology and burden of hospitalised diarrhoea

Results from the hospital-based aetiology study in Chapter Three suggest that hospitalised diarrhoea is most common in young children (8-20 months) in HCMC and is most often due to rotavirus infection (42%, 590/1,419 cases). Norovirus (17%, 241/1,419 cases) was also quite common. *Shigella* infections presenting to hospital were rare (3%, 48/1,419) though they were found in older children (median 31 months) and were often clinically severe. The isolated *Shigella* organisms showed dramatic AMR against several antimicrobials, including ampicillin and nalidixic acid; three-quarters of the *Shigella* isolates were also ESBL-producing. Though the hospital burden of *Shigella* infections may not be large in comparison to viral aetiologies, treating it appropriately in this setting may be challenging, especially considering the delay in antimicrobial susceptibility profile results (~5 days post admission) in clinical settings.

Previous estimates of *Shigella* burden from passive surveillance in hospitals in Vietnam are scarce [17], with no recent reports from HCMC. However, two hospital-based studies from the early 2000s from hospitals in Hanoi isolated *Shigella* by microbiological culture in 2.7% (48/587) and 22% (21/249) of samples from children with diarrhoea [18,19], reflecting substantial variability in prevalence. In a similar study in Cambodia, the rate of *Shigella* isolation in paediatric diarrhoeal cases presenting to hospital was 5.2% (31/600) [20]. However, hospital-seeking behaviour biases and limitations in diagnostic capacity warrant caution in interpreting these data. As in Chapter Three, molecular diagnostics were only used for viral pathogens in the referenced studies, and therefore bacterial aetiologies are likely under-reported. Given the rampant use of antimicrobials in the community in Vietnam [21], it is likely that *Shigella* organisms would be difficult to culture at hospital presentation as patients would have sought pre-treatment at a local pharmacy. Molecular diagnostics, such as the Luminex platform, may aid in generating a more accurate estimate of *Shigella* prevalence in hospitalised diarrhoeal disease in this setting.

11.3 Spatiotemporal trends of hospitalised diarrhoeal disease in HCMC

In HCMC, inner-city conditions with poor water and sanitation infrastructure are often home to the urban poor, particularly migrants lacking property rights [22–25]. Adding to this disadvantage, work from Chapter Four of this thesis demonstrates that child health in these impoverished areas of HCMC is vulnerable to the effects of climate change as children are more likely to be admitted with diarrhoea during periods of increased flooding or higher temperature. Considering the trajectory and magnitude of anticipated effects of climate change, it is likely these populations will bear a large proportion of the diarrhoeal disease burden in not only HCMC, but similar rapidly urbanising and climate-sensitive locations such as Manila and Bangkok [26,27].

Regardless of whether a vaccine is implemented in HCMC, targeted WASH interventions in regions like central HCMC would likely aid in reducing morbidity due to diarrhoeal disease in children. Large scale solutions such as improvement of water and sanitation infrastructure have been shown to be dramatically effective, yet are often impractical on a short-term scale in many impoverished regions and are only successful with committed financial government resources and political will [28–32]. Behaviour change efforts such as handwashing campaigns and household water treatment interventions, while effective in reducing diarrhoeal incidence in the short-term, also struggle to induce long lasting habit change and reduction in disease morbidity in endemic regions [33–37]. To realistically tackle WASH-challenges in a setting such as HCMC, particularly in poor communities where drinking water is often harvested through illegal taps and untreated sewage released into canals [23], sustained funding and government commitment are required [25].

11.4 Age-specific burden of diarhhoea and *Shigella* infections in the community

The burden of diarrhoeal disease in the community in HCMC remains unacceptably high, as shown through the birth cohort presented in Chapters Five and Eight. Passive surveillance for diarrhoeal disease in infants (<12 months of age) in Chapter Five led to an estimated diarrhoeal episode incidence of ~90/1000 years of follow up. However, active surveillance for diarrhoeal disease in older children (12-60 months of age) in Chapter Eight led to an estimated incidence of ~70/100 years of follow up. This order of magnitude difference in disease incidence illustrates the utility of active surveillance and suggests the incidence in infants, who were not included in the active surveillance of Chapter Eight, is likely to be much higher.

Our active surveillance incidence estimate of 70/100 CYO is substantially larger than that found in a large study conducted in the central Vietnamese coastal city of Nha Trang in the mid-2000's (11.5/100 CYO) due likely to differing strategies used to capture diarrhoeal disease [14]. This large study, by von Seidlein *et al.,* first performed education campaigns on diarrhoeal disease in the community to encourage health care attendance during a diarrhoeal episode and then relied on passive surveillance from designated local health clinics and hospitals to generate estimates of incidence [14]. Yet the overall diarrhoeal incidence estimate of Chapter Eight is lower than that of Isenbarger *et al.* who found a burden in Hanoi of 1.3 episodes/child/year in children under five years of age in the late 1990s [38], which likely reflects a true trend in declining morbidity due to diarrhoeal diseases in Vietnam over time as the country industrialises.

Data from Chapters Three and Eight show *Shigella* is a common cause of both watery diarrhoea and dysentery in hospitalised and community-based diarrhoeal in young children in HCMC. Data from the first year of the cohort study allowed for a preliminary incidence estimate of *Shigella* diarrhoeal disease of 1.5/100 CYO, which is expected to grow as the cohort matures. Our *Shigella* incidence is three times that of a large study conducted a decade ago in central Vietnam and is in fact most similar to that of Indonesia (1.9/100/year), Pakistan (1.7/100/year) and China (1.9/100/year) from the same study [14]. Interestingly, even with our relatively limited number of episodes of *Shigella* within the first year, malnourished and stunted children were still significantly more likely to report diarrhoeal disease due to *Shigella* in a nested case control study, highlighting the population most at-risk and in need of prevention and control measures in this setting. When speciation was performed in the hospital-based study in Chapter Three, *S. sonnei* was isolated in the vast majority of cases (92%), suggesting that this species continues to dominate locally.

Results from passive surveillance of diarrhoeal disease in Chapter Five highlight the burden of rotavirus in infancy, with 53% of all diagnosed samples positive for the virus. The active surveillance conducted in Chapter Eight confirms the importance of rotavirus, particularly in 12-23 month olds (7.8/100 CYO) while also highlighting the burdens of norovirus (17/100 CYO), *Salmonella* (13/100 CYO) and *C. difficile* (12.0/100 CYO) in 12-23 month olds. In older children, *Salmonella* predominated (18/100 CYO in 24-35 month olds) as well as *Campylobacter* (14/100 CYO) and norovirus (9.0/100 CYO) infections. The relative lack of *Cryptosporidium* (1.7/100 CYO) is inconsistent with the GEMS and MAL-ED studies in which it was a leading aetiology in diarrhoeal disease of young children in impoverished regions [39,40]. This difference may be due to differing ecology and risk factors of diarrhoeal disease in HCMC [41].

11.4.1 Use of molecular diagnostics

The Luminex platform has very high sensitivity and specificity for a wide range of pathogens [42–44] which allows for identification of a number of important aetiologies including ETEC and *Cryptosporidium* that are not detected through routine procedures in hospitals in Vietnam, or across other parts of Southeast Asia. If used routinely, the detection of such important pathogens may aid in not only treatment decisions but also in generation of hypotheses for further epidemiological and aetiological studies.

The use of molecular diagnostics in the active surveillance cohort must be discussed with an element of caution, however. An obvious issue raised by the high rate of coinfection in Chapter Eight and the use of the Luminex in general is pathogenicity. Platts-Mills and colleagues recently found that *Cryptosporidium*, STEC and *Shigella* were more commonly associated with severe diarrhoea than other pathogens through a birth cohort that sampled healthy stools as well as diarrheal stools [40]. However, these authors also found *Giardia*, enteroaggregative *E. coli* (EAEC), atypical enteropathogenic *E. coli* (EPEC) and norovirus G1 frequently in both diarrhoeal and healthy stool samples [40], raising the question: if a pathogen is identified in a stool sample, is it actually responsible for causing disease? If a molecular diagnostic identifies an organism in a sample, should a clinician prescribe therapy? In order to investigate relative pathogenicity in the future, use of the Luminex in combination with a quantitative technique [45], may provide more insight into the relationship between presence of a pathogen and clinical disease.

For example, *C. difficile* was identified in 15% (37/248) of stool samples from children 12-23 months of age in Chapter Eight. *C. difficile* is a significant cause of severe disease and death globally, derived often from nosocomial infection [46,47]. Yet, *C. difficile* is known to be much less common in children than adults, and asymptomatic infection with toxigenic strains is common [48]. Two studies from the 1980s suggested up to 70% of infants can be asymptomatically colonised [49,50], though the reason for the lack of disease in colonised human infants is unclear [51,52]. While use of molecular diagnostics in settings like HCMC may provide for unprecedented diagnostic scope, careful clinical consideration must be given to therapeutic implications. It is recommended that treatment should only be considered for symptomatic *C. difficile* patients [53]. Though, it has been suggested that *C. difficile* circulating in children may represent a reservoir for infection to the wider community [54], in which case treatment

for an asymptomatic infection in children may represent a potential avenue for broader control if epidemiological data from HCMC were collected to support it.

11.5 Maternal antibody dynamics in infancy

If a *Shigella* vaccine is to be considered, an important component of vaccine rollout is the determination of the vaccine schedule [55]. Given too early, a vaccine may be neutralised by the presence of maternal antibody [56,57]. Given too late and vulnerable infants are put at risk unnecessarily [58]. Work from Chapter Nine of this thesis quantifies, for the first time, the duration of maternal IgG antibody against *S. sonnei* O-antigen. The determined half-life of *S. sonnei* (43 days) is similar to that of other Gram-negative bacteria (*Haemophilus influenza* and *Bordatella*) [59,60] and represents one of the first attempts to quantify duration of persistence of maternal IgG antibody in any Gram-negative enteric bacteria [61,62]. Furthermore, Chapters Three and Five document high rates of breastfeeding in infants in HCMC, which is known to reduce both duration and severity of *Shigella* infections in young children [63–65]. Previous work has shown that secretory IgA antibody directed against the *Shigella* LPS are present in breastmilk in mothers in endemic regions and can remain for >90 days [63,66,67]. However, as demonstrated in Appendix A of Chapter Five, over half of mothers in HCMC birth cohort did not exclusively breastfeed infants beyond six months of age, indicating children could be at risk during this time period due to both lack of remaining maternally transferred serum IgG and secretory IgA.

The precise nature of the timing of a potential *Shigella* vaccine will depend on a number of factors including efficacy and safety profile in young children, route of vaccination, and number of required doses [12,68,69], but results from the thesis suggest that immunising after five months of age (and likely later) would be prudent, particularly given the disease burden is higher in two to three year olds. Recent commentary suggests that the commercial market for a *Shigella* vaccine would be improved if it was co-administered with another enteric vaccine like ETEC [12], allowing for streamlined EPI administration. Currently recommended rotavirus vaccine schedules call for the first dose at six weeks of age, coadministered with the first dose of the diphtheria/tetanus/pertussis (DTP) vaccine [70], which may be too early for a *Shigella* vaccine. The measles/mumps/rubella (MMR) vaccine is recommended to be given at 9-12 months of age, which, depending again on age-specific incidence in other areas, could be an appropriate timepoint for integration of a future *Shigella* vaccine [70]. However, it will be important to quantify the transfer and duration of *S. flexneri* serotypes as well in the event of a successful multivalent *Shigella* candidate [71].

11.6 Fluoroquinolone resistance and clinical outcome of *Shigella* infections

AMR in *Shigella*, including fluoroquinolone resistance, is an increasing problem both in Vietnam as well as globally [11,72–74]. In the event that a vaccine is unnecessary, adequate treatment will be pivotal for controlling infections and preventing onward transmission. Work from Chapter Ten set out to understand the impact of fluoroquinolone resistance on clinical outcome of shigellosis patients to better inform clinicians in this setting. The main conclusion was that *Shigella* circulating in HCMC at the time (mid- to late-2000s) were largely sensitive to fluoroquinolones and effectively treated by ciprofloxacin or gatifloxacin. The majority of isolates collected during this time period had only a single mutation in the *gyrA* gene (most commonly A87T) and had MICs below the current CLSI resistant breakpoint. Therefore, as long as *Shigella* isolates have an MIC below this threshold the infection can be effectively resolved with either ciprofloxacin or gatifloxacin.

However, since the time the isolates evaluated in Chapter Ten were collected (2006-2007), a substantial shift in AMR patterns has been observed. While only 2% of *Shigella* isolates collected in 2009 in Chapter Three exhibited full resistance to ciprofloxacin, rates have risen dramatically since. We were not able to culture all of the *Shigella* isolates collected in 2013-2014 identified by the Luminex assay in the cohort study (2/9, 22%) from Chapter Eight and thus have no antimicrobial susceptibility profile for these organisms. However, a total of 11/11 isolates collected from children in three hospitals in 2014-2015 in HCMC from an additional ongoing study were found to be fully resistant (MIC > 4µg/mL) to ciprofloxacin. These isolates, along with 49 other *S. sonnei* organisms exhibiting ciprofloxacin resistance from within and outside of Asia, were sequenced and a clonal expansion of a triple mutant (*gyrA* S83L, *parC*S80I, *gyrA*D87G) was identified across countries including Vietnam, India, Bhutan as well as travel-associated isolates in Europe, America and Australia [75]. Work from Chapter Ten demonstrates that *Shigella* harbouring *gyrA* mutations and the *qnrS* gene survive significantly longer *in vitro* in the presence of high levels of fluoroquinolone, suggesting that organisms such as the triple-mutant observed to be spreading globally may be shed into the environment for a longer period of time and outcompete sensitive strains in circulation. This rapid dominance of ciprofloxacin resistant *S. sonnei* has implications for not only treatment, but also the epidemiology of *Shigella* infections globally.

11.7 Use of a *Shigella* vaccine in Vietnam

The persistence and potential growth of diarrhoeal morbidity in areas such as HCMC is difficult to remedy. Vaccines are without a doubt one of the most effective tools of modern public health and may play a major role in diarrhoeal prevention globally. Yet with only eight enteric vaccines licensed to date, covering only four pathogens (poliovirus, *Salmonella* Typhi, rotavirus and *Vibrio cholerae*) the development of future enteric vaccines, particularly mucosal vaccines, is a significant challenge [76–78]. It is a challenge worth meeting, however, given the alarming rise in AMR among bacterial organisms such as *Shigella*, which threatens to reverse progress made reducing diarrhoeal disease burden in recent years [79–83].

Whether a vaccine for *Shigella* should be developed generally is not in doubt. The global morbidity and mortality of disease combined with rapidly expanding AMR warrants the significant time and investment required for licensure [39,40,84]. Indeed several promising candidates are in clinical trials currently [85,86]. However, determining whether a *Shigella* vaccine is necessary for HCMC at this time is not straightforward. While the documented burden in 2-5 year old children (1.5 episodes/100 CYO) is relatively substantial and similar to burdens in other Asian countries [14], the more significant burdens of other aetiologies of diarrhoeal disease identified in this thesis, particularly rotavirus, mandates careful consideration of available funding and political attention toward any future *Shigella* vaccine. In the context of the current healthcare funding situation in Vietnam, with limited financing generally and extremely strained resources as it stands [87,88], it is unlikely that a *Shigella* vaccine is worth the expense and effort for the limited burden of disease documented in Chapter Ten. A formal cost-effectiveness study is warranted prior to any firm conclusions [89], but the relatively low morbidity documented in this thesis predicts a *Shigella* vaccine programme is not justified at this time.

Continued surveillance, both epidemiological and molecular, are necessary however. While fluoroquinolones and third generation cephalosporins may currently be effective in the majority of patients presenting to hospital in this setting, this is not likely to remain the case [11]. In the event of fully AMR *Shigella* circulating in HCMC, vaccination may need to be reconsidered. Furthermore, if Stx-producing *Shigella* begin to circulate as has been observed in Haiti and the USA [15,16], the clinical severity of these infections may again warrant reconsideration of vaccination. Ensuring that existing surveillance systems are robust and timely is a worthy investment to keep abreast with this rapidly evolving pathogen.

As documented in Chapters Three, Five and Eight, a majority of the burden of hospitalised and community-based diarrhoea in HCMC is due to rotavirus, particularly in the first and second years of life. However, as shown in Chapter Eight, only 40% of children received at least one dose of RoV vaccine in HCMC. Instead of pursuing a *Shigella* vaccine currently, it is recommended that Vietnam use existing resources to continue to bolster rotavirus vaccine uptake to limit the burden of diarrhoeal disease. Though expensive (US$70-80), previous research has indicated that the vaccine would be cost-effective in Vietnam if GAVI-subsidised [90] in addition to being safe when co-administered within the current PEI schedule [91]. Furthermore, Vietnam is working to develop a naturally attenuated monovalent GIP[8] vaccine candidate to produce locally in an effort to reduce the cost [92]. Using the current national health budget to encourage rotavirus vaccination would likely have a larger impact on childhood diarrhoeal morbidity than attempting to vaccinate against shigellosis.

Although the advent of rotavirus vaccines will undoubtedly continue to reduce morbidity and mortality [93,94], prevention and control in the post-rotavirus vaccine era is not straightforward, as Platts-Mills and colleagues have suggested [40]. Non-vaccine based strategies will need to be pursued to prevent and control *Shigella* infections in HCMC. Breastfeeding is known to be protective against shigellosis, and to reduce severity and duration of infection in infants [64]. Yet aggressive marketing of milk formulas and supplements has led to a drop in prevalence and duration of breastfeeding in Vietnam [95], which may lead to a corresponding lack of protection against pathogens such as *Shigella* in this population. Therefore encouragement of exclusive breastfeeding for as long as possible is important in HCMC. WASH-based interventions are also effective [28–32], although again required sustained funding and political support. Finally, optimising therapy, particularly in the face of growing fluoroquinolone resistance, will help to prevent onward spread of infection.

11.8 Limitations

One of the major limitations of this thesis is the lack of generalisability of data collected in HCMC to greater Vietnam. HCMC is the country's largest, wealthiest city, and although it has a large number of poor, inner-city slum residents [96], the demographics and urbanised nature of the city make it dissimilar to the majority of the country [97]. In Chapter Five, for example, the rates and aetiologies of diarrhoeal disease were significantly different between urban HCMC and rural Dong Thap province (120km southwest of HCMC). Though Dong Thap and the surrounding the Mekong River Delta region (one of eight in the country) have a higher rate of poverty (10.3%, defined as a

per capita expenditure of less than US$10/month) compared to the Southeast Region of HCMC (5.8%), there are regions in Vietnam such as the Northwest Mountains (49%) and Central Highlands (29%) that have far greater levels of poverty [98]. So although the burden of *Shigella* in HCMC is not substantial enough to justify a vaccine programme, the epidemiological situation of diarrhoeal disease is likely to be dramatically different outside of HCMC. Whether a *Shigella* vaccine is warranted in rural Vietnam cannot be addressed through data in this thesis.

An additional limitation includes the lack of diagnostic consistency across the different studies included in the thesis. Ideally, the Luminex platform would have been available for use at all timepoints to allow for consistent comparisons. However due to the expense (US$75/sample) this was not feasible. Finally, as shown in Chapter Eight, the bulk (57%) of diarrhoeal episodes are not seen in hospital in HCMC. Therefore it is likely that the majority of the work presented in this thesis is biased by healthcare-seeking behaviour. The reported results, particularly for Chapters Three, Four and Five, therefore pertain to those who are able to seek healthcare and who are likely wealthier and epidemiologically otherwise different to those children and families who could not attend healthcare. The cohort in Chapter Eight and relevant data in Chapter Nine should not be as biased due to active surveillance. Additionally, though considerable effort was made to collect disease information, we cannot guarantee that we were able to capture every diarrhoeal episode throughout the cohort, which may lead to an underestimated incidence measurement. Regardless, the work presented in this thesis presents the most comprehensive epidemiological evaluation of diarrhoeal disease and *Shigella* in HCMC to date, and is likely representative of other rapidly developing cities in the region.

11.9 Alternative approaches and future directions

Several alternative approaches to determine the epidemiology of diarrhoeal disease and evaluate the need of a *Shigella* vaccine could have been employed. Active surveillance in infancy would have allowed for more robust incidence calculations for an age group where infection can often be severe [9]. Additionally, though there is evidence of a demand for a vaccine against shigellosis/dysentery from parents of young children in East Asia [99], an evaluation of local physician attitudes toward the need for such a vaccine in this setting would have led to useful insight on the burden and perceived severity of the syndrome by local healthcare workers. Such work would have broadened the scope of the evaluation beyond just the hospital and districts directly included in the thesis.

Common transmission pathways of *Shigella* in the HCMC context remains an unanswered question. Whether transmission occurs predominately via person-to-person contact [100–103], via contaminated water supplies [104,105] or a combination of both is unclear. Two additional avenues of investigation would aid in generating evidence to answer this question. First, environmental sampling of local drinking water supplies would permit identification and quantification of *Shigella* DNA, as was done to investigate the prevalence of organisms causing typhoid fever in Kathmandu, Nepal [106]. Sampling of a variety of water sources, such as wells, the municipal supply and purchased bottled water is recommended. To investigate person-to-person transmission, a study evaluating in-household transmission of *Shigella* would be invaluable. By identifying index cases in hospital and subsequently enrolling and sampling family members, secondary attack rates could be estimated [107]. Whole genome sequencing of organisms, both from water supplies as well as from in-household transmission investigations, would allow for more robust conclusions as to likely transmission pathways [108]. An enhanced understanding of transmission pathways would aid in targeting non-vaccine based interventions in HCMC.

In the future, additional investigations into the epidemiology of diarrhoeal disease and *Shigella* in locations in Vietnam other than HCMC would be beneficial. Study sites in rural locations, for example, or in predominately minority regions would help to better inform policy decisions regarding diarrhoeal disease for the country as a whole. There is a large, ongoing study through the Oxford University Clinical Research Unit (OUCRU) that is examining the aetiology and prevalence of a number of common syndromes in Vietnam, including diarrhoeal disease, in six different locations to investigate zoonotic infections [109]. With over 3,000 diarrhoeal samples projected to be collected across a number of epidemiologically variable locations, such a study presents an unprecedented opportunity to examine geographic and socioeconomic differences in the aetiology of diarrhoeal disease in Vietnam. Furthermore, this data will likely highlight areas at increased risk for *Shigella* infections in which future research could take place.

Next, evaluating the prevalence of diarrhoeal pathogens in non-diarrhoeal stool samples from the cohort will provide important evidence on the relative pathogenicity of organisms identified by the Luminex platform. As mentioned, both the GEMS and MAL-ED studies identified a number of aetiologies (aEPEC, *Giardia* etc) frequently in healthy stool [39,40]. A pathogenicity index for HCMC specifically will help to inform clinical therapeutic decisions in this setting. Furthermore, the cohort will provide for an evaluation of longitudinal immune responses to a number of *Shigella* antigens in children which can aid in informing future vaccine trials [110].

Finally, one of the directions for future research derived from this thesis is the need for rapid diagnostics that are universally accessible [111]. Doctors in public hospitals in HCMC still rely on microbiological culture and subsequent drug sensitivity testing, which can take up to five days to complete. As the median length of stay of children with diarrhoea in hospitals in HCMC is five days [112], the need for faster diagnostics is clear. The low sensitivity of microbiological culture demonstrated in Chapter Eight is likely due to high use of antimicrobials in the community prior to clinical consultations [21,113], and calls for more sensitive techniques to be employed. An ideal enteric diagnostic test for settings such as HCMC would need to be inexpensive, easy to use, fast, refrigeration-free, transportable, cover a wide variety of pathogens and have high sensitivity and specificity [114,115]. Effective multiplex molecular techniques, such as the Luminex platform, are available [44,116–118]. However, the cost (US$75/reaction) of the Luminex platform is prohibitive for government hospital use in HCMC. Some progress has been made into techniques, such as PCR, that have been adapted for low-resource settings [119]. With a lack of a vaccine and large hurdles in improving socioeconomic status, identification and prompt and appropriate therapy is currently one of the most feasible manners in which to reduce morbidity and transmission due to *Shigella* in endemic areas.

11.10 Conclusions

The cohort study developed in this thesis will continue to provide important information regarding *Shigella* diarrhoeal disease in an industrialising setting as the children mature. A detailed risk factor analysis during the period of known burden of *Shigella* infections (two to three years of age) with a full, complete dataset is hoped to provide specific information on potential routes of transmission to generate tangible and targeted prevention activities. It is known that *Shigella* is commonly transmitted person-to-person [10,120,121], but through the cohort we are aiming to identify the important groups involved in transmission, whether it be in schools, between older or younger siblings in the home or potentially due to contact with grandparents, who are often the caretakers of children in HCMC and have been found to present commonly with *Shigella* in China [122]. An integrated approach using evidence-based prevention measures, including promotion of breastfeeding in the community in HCMC would serve to prevent morbidity due to a variety of diarrhoeal pathogens, including *Shigella* [123], in the absence of a vaccine programme.

In conclusion, diarrhoeal disease is a public health challenge in HCMC. Low rotavirus vaccine uptake and a dramatic increase in antimicrobial resistance against first- and

second-line therapies in bacterial agents such as *Shigella* are driving the continued persistence of morbidity in young children in this setting. Affordable and accurate diagnostics and targeted prevention measures in settings such as HCMC would help to alleviate short- and long-term suffering due to diarrhoeal disease in young children, particularly among poor, inner-city communities. A future *Shigella* vaccine, though not necessary in HCMC at this time, would greatly aid in reducing the burden of disease globally, particularly in the face of rampant AMR.

11.11 References

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12 MANUSCRIPTS

During the period of my PhD (2012-2015) I contributed to the following manuscripts related to diarrhoeal disease in children in Vietnam:

1. Vien LTM, Minh NNQ, Thuong TC, et al. The co-selection of fluoroquinolone resistance genes in the gut flora of Vietnamese children. PLoS One **2012**; 7:e42919.

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